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PROCEEDINGS
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DEVELOPMENTAL CHANGES IN APICAL MERISTEMS

By W. Gordon Whaley

Department of Botany, Columbia University

Communicated July 20, 1939

The primary meristem is essentially the functional embryo of a plant. It remains undifferentiated, continually gives rise to new cells and is the ultimate source of all the organs of the plant. It has been studied for the most part in relation either to phyllotaxis or to the problem of germ layers. The significance of this meristem in the ontogeny of the plant has been stressed, but little attention has been given to ontogenetic changes within the meristem itself. Such changes obviously underlie its rôle in plant development. In an attempt to determine what changes take place in the apical meristem of the shoot during post-embryonic growth and their relation to growth of the plant as a whole, a series of developmental studies was made of these meristems in three species of tomato, *Lycopersicon esculentum*, *L. racemigerum* and *L. pimpinellifolium*.

Methods.—Plants of each of the three species were grown under normal field conditions. At uniform intervals during development main stem tips of each type were killed and fixed. These, together with embryos from the seeds of each of these species, were imbedded in paraffin and cut into longitudinal serial sections, mounted and stained. The volume of the apical meristem, which for the purpose of these experiments is defined as that region at the stem tip in which the cells are meristematic, non-vacuolate and essentially isodiametric, was determined as follows: Projection drawings were made of each of the serial sections. These drawings were then placed upon wax plates of uniform thickness (determined by the thickness of the original sections and the magnification used) and cut out in the wax with a steel needle. Thus a wax model of each of the original sections was obtained. By weighing together those models corresponding to the series of sections through each stem tip, multiplying the weight by the specific

gravity of the wax and correcting for magnification, the volume of each meristem could be calculated.

Cell volumes were obtained from the median section of each series. Camera lucida drawings were made of as many meristem cells as possible, exclusive of those in the dermatogen. The average of the longest and the shortest diameters of each of the drawings was determined and divided by the magnification. This result was cubed, giving a measure of cell volume adequate for comparable purposes.

Since meristem nuclei are spherical, their volume could be determined simply by making camera lucida drawings as for the cells, measuring the diameters, dividing by the magnification and substituting the result in the formula for the volume of a sphere.

Apical Meristem Volume.—By the technique outlined above it was possible to measure the actual volume of the meristem. The results are given in table 1.

TABLE 1

MERISTEM VOLUME IN CUBIC MICRA

	L. PIMPINELLIFOLIUM	L. RACEMIGERUM	L. ESCULENTUM
Embryo	249,000	506,000	1,246,000
14 days	296,000	822,000	2,043,000
35 days	483,000	932,000	2,574,000
56 days	859,000	1,059,000	2,205,000
77 days	622,000	1,221,000	2,305,000
98 days	543,000	1,343,000	1,763,000

There is evidently a marked increase in the volume of the meristem during development. In *pimpinellifolium* and *esculentum* there appears to be a subsequent decrease. It was observed that growth continues considerably longer in *racemigerum* than in either of the other species, a fact which may be related to the absence of any decrease in meristem size in this species. In the others this decrease takes place generally at the point at which the growth curve begins to level off.

The volume of the apical meristem is correlated directly with the size of the determinate organs which it produces. This correlation holds not only for the plumular meristem in the seed but also for any given stage during development. Table 2 gives representative measurements of certain organs of each of the species.

TABLE 2

COMPARATIVE ORGAN SIZE

	L. PIMPINELLIFOLIUM	L. RACEMIGERUM	L. ESCULENTUM
Leaf length	13.8 ± 0.36 mm.	23.3 ± 0.27 mm.	24.2 ± 0.21 mm.
Leaflet length (second basal)	3.66 ± 0.09 mm.	6.93 ± 0.13 mm.	8.32 ± 0.13 mm.
Corolla width	10 mm.	19 mm.	34 mm.
Fruit weight	1.8 ± 0.01 gm.	11.4 ± 0.11 gm.	93.4 ± 1.8 gm.

L. pimpinellifolium, with the smallest apical meristem (table 1), has the smallest organs; *L. esculentum* has the largest meristem and the largest organs; and *L. racemigerum* is intermediate with respect to both meristem and organ size. This correlation between organ size and meristem size is of considerable significance in respect to the inheritance of organ size, since it indicates that the factors controlling organ size differences do so by determining the meristem size.

Meristem Cell Size.—Rössler[1] working with *Triticum vulgare*, and Murneek and Gomez[2] with *Soya max* found some evidence for a reduction in the size of the meristem cells during development. The present more detailed studies with *Lycopersicon* (table 3) furnish substantial proof that a progressive diminution in the size of the meristem cells does take place as growth proceeds.

TABLE 3

VOLUME OF CUBIC MICRA OF MERISTEMATIC CELLS AND NUCLEI DURING DEVELOPMENT

	L. PIMPINELLIFOLIUM		L. RACEMIGERUM		L. ESCULENTUM	
	CELL	NUCLEUS	CELL	NUCLEUS	CELL	NUCLEUS
Embryo	1082±91	66±1	1394±80	69±2	1331±69	103±5
14 days	697±28	76±6	588±40	87±2	521±37	89±3
35 days	558±25	71±3	406±13	73±2	396±18	77±2
56 days	396±16	59±3	333±9	59±1	336±7	68±2
77 days	327±25	72±2	305±6	48±2	308±9	56±1
98 days	342±25	73±3	288±7	58±2	295±6	57±2

The progressive decrease in cell size indicates that cell division is proceeding faster than cell enlargement in the meristem, resulting in the failure of the daughter cells to equal the size of the mother cell before dividing again. This condition obtains only during the period of active growth. Maturity is preceded or accompanied by the attainment of a minimal cell size, which then seems to remain constant. Further experiments are necessary to determine how general this situation is in annual plants, but in tomato, at least, development from the seed embryo to the mature plant is accompanied by a marked progressive diminution in the size of the meristem cells, which seems to be a result of a changing balance between the rate of division and the rate of protoplasmic synthesis. Presumably any environmental factor, such as a change in the photoperiod, which disturbs this balance will also affect the time of onset of maturity. This reduction in cell size is an indication of the slowing down of synthesis of new material and is thus related to cessation of growth.

Meristem Nuclear Size.—The nuclei (table 3) of the apical meristem cells behave in much the same manner as the cells during development. As growth proceeds there is a progressive diminution in nuclear volume, a constant minimal volume being reached, as with the cells, at the time of flattening off of the growth curve. The nuclei, however, decrease at a much

less rapid pace than do the cells, and as a result the small cells characteristic of the later growth stages have relatively larger nuclei and less cytoplasm than the large cells found in the plumular and young seedling meristems. Thus the changes in cell/nucleus ratio may be concerned in bringing about the cessation of growth.

Summary.—Ontogenetic changes in the apical meristems of three species of *Lycopersicon* are described. Meristem volume tends to increase during development. The size of the meristem is correlated with the size of the determinate organs which it produces. Apical cells and their nuclei show a progressive diminution in size, a constant minimal size being attained with the onset of maturity. This change is evidently related to the annual growth cycle of the plants.

[1] Rössler, P., *Planta*, **5**, 28–69 (1928).

[2] Murneek, A. E., and Gomez, E. T., *Mo. Agr. Exp. Sta. Res. Bull. 242* (1936).

THE SYNTHESIS OF SULFONYL CHLORIDES BY CHLORINATION OF SULPHUR COMPOUNDS

BY TREAT B. JOHNSON

STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY

Communicated July 19, 1939

Introduction.—Reference is being made daily by scientific and technical journals and the public press to the organic compounds—*sulfanilamide* and *sulfanilylaminopyridine*—whose introduction in medicine is one of the most valuable contributions by chemistry to this profession in recent years. The therapeutic side of the sulfanilamide type of sulphur compounds is now in a primitive state of development, and we are really at the beginning of a new chemotherapy today calling for close collaboration between chemists, biologists and clinicians. This discovery of the therapeutic value of these sulphur constructions emphasizes once more how the organic synthetic products of our research institutions and chemical industry are influencing the lives of our people.

The evolution of chemical reactions applicable for the practical synthesis of organic sulfonyl chlorides and amides has been receiving the attention of workers in this laboratory since the publication of a paper by James M. Sprague and the author, in 1935, describing the action of chlorine-water on certain 2-mercaptopyrimidines.[1] In a footnote inserted in that publication (p. 2253) the authors wrote as follows: "the mechanism of these transformations is being investigated and the results will be discussed in a future paper." Within this period of time ten publications

have been contributed to the press on this subject from this laboratory,[1] and three United States Patents have been granted, which are based on the results of our investigations in this field.[2]

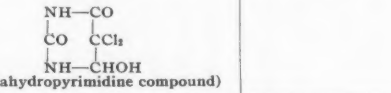
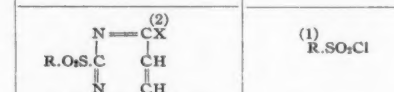
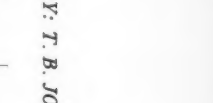
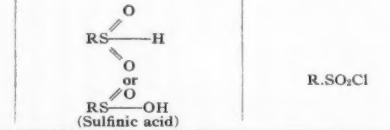
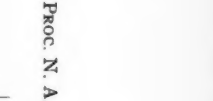
Chlorination Reactions.—The synthetic operations that have received chief attention in our researches on the oxidation of sulphur compounds have been confined to experimentation designed to determine the reactivity of chlorine with sulphur compounds characterized constitutionally by an isothiourea or related structure. The classification of sulphur compounds on which we will report briefly in this paper embraces three types of organic compounds, namely, isothioureas, 2-mercaptopyrimidines or isothioureas in cyclic combination and thiocyanates. In order to conserve space by omitting quotation of experimental data previously published, a condensed tabulation of the results of our experiments on chlorination of these three types of sulphur compounds is recorded in table 1. A knowledge of the many details and the technique of experimentation can be acquired by consulting the series of publications registered in the literature.[1]

An inspection of table 1 will suggest the fact that we are dealing apparently in all these oxidation changes brought about by the action of chlorine water with a common reaction mechanism. The outstanding results obtained are (*a*) that a sulfonyl chloride (RSO_2Cl) is the characteristic end-product containing sulphur of the chlorination reaction, and (*b*) that the nitrogen-containing nucleus of the original sulphur compound exposed to the action of chloride retains a constitution which is easily identified. In fact, it has been our experience that the yields of sulfonyl chlorides from both isothioureas and thiocyanates are good, and that either type of sulphur compound may serve as practical key reagents for the synthesis of aliphatic sulfonyl chlorides.

Mechanism of Reaction.—It is our conclusion that the first reaction step in this chlorination operation is the addition of oxygen to sulphur to form a sulphoxide intermediate as expressed by formula (I). This applies to all three types (*A*, *B* and *C*) represented in table 1. We have practically no knowledge of the chemical properties of sulphur constructions of this type, but it would be predicated, however, that such a sulphur configuration with a sulphur valency of 4 would be unstable in the presence of a strong oxidizing agent like hypochlorous acid. Such a sulphur grouping would be expected to undergo a structural change by interaction with chlorine water to give either (*a*) a sulphone (II) by further oxidation or (*b*) undergo hydrolysis with cleavage of the linkage between sulphur and carbon with production of a sulfinic acid (III) or a sulfinyl chloride (IV), and a stable nitrogen-containing nucleus. It is well known that sulfinic acids are easily oxidized by chlorine water to sulfonic acids and interact with hypochlorous acid to form sulfonyl chlorides (V).[3] The sulfinyl compounds (IV) are characterized by their instability and easily undergo oxida-

TABLE 1

CHLORINATION OF ORGANIC SULPHUR COMPOUNDS IN THE PRESENCE OF WATER

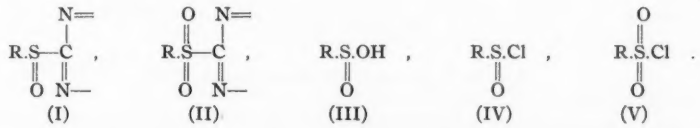
TYPES OF SULPHUR COMPOUNDS CHLORINATED	TYPES OF ORGANIC SULPHUR COMPOUNDS FORMED I	TYPES OF ORGANIC SULPHUR COMPOUNDS FORMED II	ORGANIC NITROGEN RESIDUE FORMED	INTERMEDIATES
A 2-MERCAPTOPYRIMIDINES				
(2-Mercaptodihydropyrimidines)	(1) $R.SO_2OH$ (Sulfonic acid)		 (Hexahydropyrimidine compound)	
(2-Mercaptopyrimidines)	 (2-Sulfone-pyrimidine)	(1) $R.SO_2Cl$		(Sulfinyl chloride)
B ISOTHIOUREAS				
(1) *1.* $RS.C(NH_2):NH.HCl$ (*N*-unsubstituted)		$R.SO_2Cl$	NH_2 | $Cl.C:NH.HCl$ or $NH_2CN.2HCl$ (Cyanamide dihydrochloride)	
2. $RSC(NHCH_3):NH.HCl$ (*N*-monosubstituted)		$R.SO_2Cl$	$CH_3NHCN.HCl$ (Methylcyanamide hydrochloride)	
3. $RSC(NHCH_3):NCH_3.HCl$ (*N,N'*-disubstituted)		$R.SO_2Cl$	$CH_3NHCONHCH_3$ (Dimethylurea)	
4. $RSCN(CH_3)_2:NCH_3.HCl$ (*N,N,N'*-trisubstituted)		$R.SO_2Cl$	$(CH_3)_2N.CONHCH_3$ (Trimethylurea)	
C THIOCYANATES				
(1) $R.SCN$	 (Sulfinic acid)	$R.SO_2Cl$	$CN.Cl$ (Cyanogen chloride)	

(1) R = alphyl or aryl radicals. The alkylated isothioureas are all expressed as methyl derivatives (CH_3) for simplicity, and this does not mean that only methylated ureas undergo the changes represented. The only intermediates represented are the sulfinylchlorides which undergo oxidation very easily to form sulfonylchlorides, $R.SO_2Cl$.

(2) X = H, halogen, alkyl, $—OCH_3$, NH_2, etc.

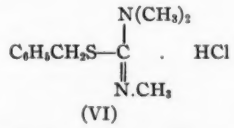
tion even in contact with air to give the corresponding sulfonyl chlorides (V).[4] An inspection of table 1 will reveal the fact that representatives of the three types of sulphur degradation products (II), (III) and (V) are recorded under the heading "types of organic sulphur compounds formed."

The experimental results, therefore, furnish evidence in support of the postulation that the sulfinyl chlorides (IV) are intermediate products of these oxidation reactions, and serve as the precursors of the sulfonyl chlorides (IV) obtained as final reaction products. It is particularly important to note here that sulfonic acids do not interact with hypochlorous acid to form sulfonyl chlorides.[5]



(I) (II) (III) (IV) (V)

Attention is called to the striking difference in behavior of 2-mercapto-dihydropyrimidines and 2-mercaptopyrimidines, so far examined, towards chlorine water leading to the formation of a hexahydro-pyrimidine and free sulfonic acid, and true sulfone derivatives, respectively. These characteristic reactions serve as a clean-cut method for differentiating between these types of 2-mercaptopyrimidine structures. No reference has been made in any of our previous publications to the behavior of *N,N,N'*-trisubstituted isothioureas towards chlorine water. We wish to report here that the hydrochloride of the isothiourea (VI) interacts



(VI)

smoothly to yield benzylsulfonyl chloride and trimethylurea (m. p. 74–75°).

(1) R = alphyl or aryl radicals. The alkylated isothioureas are all expressed as methyl derivatives (CH_3) for simplicity, and this does not mean that only methylated ureas undergo the changes represented. The only intermediates represented are the sulfinylchlorides which undergo oxidation very easily to form sulfonylchlorides, $R.SO_2Cl$.
(2) X = H, halogen, alkyl, —OCH_3, NH_2, etc.

[1] *Sequence of publications on Chlorination:* Sprague, J. M., and Johnson, T. B., *Jour. Amer. Chem. Soc.*, **57**, 2252–2255 (1935); Johnson, T. B., and Sprague, J. M., *Science*, **83**, 528–530 (1926); Sprague, J. M., and Johnson, T. B., *Jour. Amer. Chem. Soc.*, **58**, 423–426 (1936); Johnson, T. B., and Sprague, J. M., *Ibid.*, **58**, 1348–1352 (1936); Sprague, J. M., and Johnson, T. B., *Ibid.*, **59**, 1837–1840 (1937); Douglass, I. B., and Johnson, T. B., *Ibid.*, **60**, 1486–1489 (1938); Johnson, T. B., and Sprague, J. M., *Ibid.*, **60**, 1622–1624 (1938); *Ibid.*, **61**, 176–179 (1939); Johnson, T. B., and Douglass, I. B., *Jour. Amer. Chem. Soc.* (unpublished).

[2] *United States Patents:* No. 2,146,744 (Feb. 14, 1939), "Process of preparing sulfonyl halides and sulfonic acids from pseudothioureas"; No. 2,147,346 (Feb. 14, 1939), "Sulfonic halides"; Serial No. 201,728, Filed in U. S. Patent Office Apr. 13, 1938; Application allowed March 7, 1939.

[3] List, R., and Stein, M., *Ber.*, **31**, 1648–1672 (1898); Otto, R., and Tröeger, J., *Ibid.*, **24**, 478–488 (1891); Otto, R., *Ann.*, **145**, 317–329 (1868); Otto, R., and Ostrop, H., *Ibid.*, **141**, 365–377 (1867).

[4] Hilditch, T. B., and Smiles, S., *Ber.*, **41**, 4113–4116 (1908); Spring, W., and Winsinger, C., *Ibid.*, **15**, 445–448 (1882); *Ibid.*, **17**, 537–540 (1884); *Bull. Soc. Chim.*, (2) **49**, 68–74 (1887).

[5] Spring, W., and Winsinger, C., loc. cit.

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*HYBRIDIZATION AND TUMOR FORMATION IN MICE**

By C. C. Little

Roscoe B. Jackson Memorial Laboratory

Communicated July 20, 1939

The object of this paper is to record the incidence of tumors in a species cross in mice and to discuss their frequency in that cross in relation to their frequency in the parent strains.

The two strains used differed from one another in size, rate of growth, fertility and tumor incidence. One strain was the inbred descendants of wild *Mus bactrianus* obtained from China in 1926 and reported on by Green in a series of papers (1931) (1935).

Mus bactrianus

	TOTAL MICE	MICE WITH TUMORS	% WITH TUMORS	NO. OF TUMORS	TOTAL TUMORS / TOTAL ANIMALS	NO. OF MICE WITH EPITHELIAL TUMORS	% EPITHELIAL TUMORS	NO. OF MICE WITH NON-EPITHELIAL TUMORS	% NON-EPITHELIAL TUMORS
Males	20	2	10.0	2	10.0	2	10.0	0	0.0
Females	139	4	2.9	4	2.9	4	2.9	0	0.0
Combined Sexes	159	6	3.8	6	3.8	6	3.8	0	0.0

It will be noted that all six tumors were epithelial in origin.

It is also worth mentioning that no animal had more than one tumor. This will be in marked contrast with the other material.

The second parent strain, *C57* black, is an inbred derivative of *Mus musculus*. This strain has been extensively used in cancer research and has been described by several investigators (Murray and Little, 1935) (Little, Murray and Cloudman, 1939).

The animals of this strain are large and fertile. They mature more rapidly than do *Mus bactrianus*. Their tumor incidence is as follows:

C57 BLACK MICE

	TOTAL MICE	MICE WITH TUMORS	% WITH TUMORS	NO. OF TUMORS	TOTAL TUMORS / TOTAL ANIMALS	NO. OF MICE WITH EPITHELIAL TUMORS	% EPITHELIAL TUMORS	NO. OF MICE WITH NON-EPITHELIAL TUMORS	% NON-EPITHELIAL TUMORS
Males	174	32	18.4	36	20.7	4	2.3	31	17.8
Females	703	94	13.4	101	14.4	10	1.4	85	12.1
Combined Sexes	877	126	14.4	137	15.6	14	1.6	116	13.2

It will be noted that non-epithelial tumors are distinctly more frequent in this strain than are epithelial tumors. Neither strain is high in epithelial tumors, however, the incidence being 3.8% for *M. bactrianus* and 1.8% for *C57* black.

First generation hybrids were made by crossing *C57* black females with *Mus bactrianus* males. These F_1 animals were not allowed to breed but were observed carefully for tumor incidence. The results are as follows:

F_1 *C57* BLACK × BACTRIANUS

	TOTAL MICE	MICE WITH TUMORS	% WITH TUMORS	NO. OF TUMORS	TOTAL TUMORS / TOTAL ANIMALS	NO. OF MICE WITH EPITHELIAL TUMORS	% EPITHELIAL TUMORS	NO. OF MICE WITH NON-EPITHELIAL TUMORS	% NON-EPITHELIAL TUMORS
Males	59	21	35.6	27	45.8	9	15.2	17	28.8
Females	62	34	54.8	45	72.6	7	11.3	31	50.00
Combined Sexes	121	55	45.5	72	59.5	16	13.2	48	39.66

There is in the F_1 a striking increase of all types of tumors which can be brought out by the following tabulation:

	% WITH TUMORS	TOTAL TUMORS / TOTAL ANIMALS	% EPITHELIAL TUMORS	% NON-EPITHELIAL TUMORS
M. bactrianus	3.8	3.8	3.8	0.0
C57 black	14.4	15.6	1.8	13.8
F_1 hybrids	45.5	59.5	17.4	42.1

Discussion.—The increase in tumor incidence is too great to be explained by any simple genetic formula. If one *adds* the tumor forming tendencies of both parent strains, he still falls far short of the record made by the F_1 hybrids. Very evidently there are other influences than those of routine genetic type, which must be considered.

The question of age will naturally be one of importance. An attempt to correct for this factor has been made in calculating the percentage of tumor incidence. In each generation the age of the animal at the appearance of

the first malignant tumor has been used as a threshold. Animals dying without tumors below this age have been excluded from the tabulation. This makes each generation comparable in so far as method of calculating the tumor incidence is concerned. While any method leaves something to be desired, the one employed is as nearly satisfactory as any that can be used with the number of animals available.

In order, however, to obtain another check on the effect of age, a different method can be employed. Here the age span at which tumors occur in the three groups can be compared.

	AGE AT FIRST TUMOR	AGE AT LAST TUMOR
M. bactrianus	390 d.	944 d.
C57 black	271 d.	1185 d.
F_1 hybrids	565 d.	1166 d.

Obviously there is no significant difference between the extreme upper age limits in *C57* black and F_1 hybrids. The only possible discrepancy is in the upper age limit of the *bactrianus* stock. There were non-tumor animals of that stock which survived more than 1100 days. In order to give the least favorable aspect of tumor increase, however, the F_1 tumors occurring later than 944 days can be eliminated. When this is done the tumor incidence in the hybrids still remains at 28.1% as compared with 3.8 in *bactrianus* for the same age range.

We may, therefore, conclude that as a result of hybridization there has been a great increase in incidence of both epithelial and non-epithelial tumors.

Not only is this an actual increase in number of tumors, but the tendency to tumor formation within the individual has been made greater. This can be shown by the following tabulation:

	ANIMALS WITH ONE TUMOR	ANIMALS WITH TWO TUMORS	ANIMALS WITH THREE TUMORS	ANIMALS WITH FOUR TUMORS	% OF TUMOR MICE WITH MULTIPLE GROWTHS	% OF POPULATION WITH MULTIPLE TUMORS
M. bactrianus	6	0	0	0	0.0	0.0
C57	116	10	0	0	7.9	1.14
F_1 hybrids	42	10	2	1	23.6	11.57

The occurrence of multiple growths is approximately three times as frequent in the hybrids as in the *C57* black stock. There were no multiple tumors in *bactrianus*. The existence of two hybrids with three different types of primary tumor and of one with four types finds no counterpart in either parent stock.

There exists in the work of Gordon (1927) (1938) a parallel with the present situation. Working with species of two genera of small tropical fishes he found that the hybrids between them produced large numbers of melanotic neoplasms absent in both parent species.

Evidently the presence in the hybrids of conflicting tendencies of growth

and organization produced conditions under which controlled growth and orderly development of pigment metabolism were seriously upset.

The present cross with mice is the first in mammals to provide a roughly analogous situation. Where other such crosses have been made the hybrid animals have not been kept under observation for tumor incidence throughout their whole life span.

The present evidence, therefore, indicates that the mammalian body, in which marked physiological imbalance exists as the result of the presence of widely different growth potentialities, is a medium in which the incidence of uncontrolled growth is greatly increased.

Balance of the internal environment becomes, then, an all-important factor in maintaining orderly and controlled growth of its constituent tissue elements. This evidence is entirely consistent with the rôle which imbalance in hormonal secretion has played in tumor formation. It also is in agreement with the theory advanced by Murphy that a balance between activating and inhibiting agents within the body is essential to the maintenance of controlled growth.

Incidentally, it offers further support for the belief that the origin of centers of neoplastic growth is a complex matter dependent in various tissues upon a characteristic comb nation of chemical substances, some of which stimulate, and others of which inhibit cell division.

By the extension of experimentation involving hybridization of distinctly different strains and species, we should be able to add significantly to our knowledge of the nature of tumor formation in mammals.

Summary and Conclusions.—1. A cross between two species of mice which differ markedly from one another in size, fertility and rate of growth has greatly increased the incidence of tumors in the first generation hybrids.

2. The increase consists not only of more tumors but in a greater tendency to form multiple tumors in individual mice.

3. Internal physiological imbalance increased by the process of hybridization is the most reasonable explanation for the added tumor incidence.

4. Hybridization as an experimental approach should prove a fruitful method of analyzing the factors of the internal environment which contribute to tumor formation.

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NUCLEASE ACTION, PROTEASE ACTION AND HISTOCHEMICAL TESTS ON SALIVARY CHROMOSOMES OF DROSOPHILA

BY DANIEL MAZIA AND LUCENA JAEGER

DEPARTMENT OF ZOÖLOGY, UNIVERSITY OF MISSOURI

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There is considerable evidence for the presence of both nucleic acids and proteins in the salivary gland chromosomes of certain diptera. The so-called "euchromatic" bands stain intensely after application of the Feulgen-Rossenbeck technique, which is a fairly specific test for the pentose of animal nucleic acids. The heterochromatic regions also give a weak reaction, and the boundaries between the bands are not sharp.[1] Caspersson[2] found that the material of the euchromatic bands had a similar ultra-violet absorption spectrum to the nitrogenous bases characteristic of nucleic acids. There is little doubt, therefore, that the euchromatic bands are regions of high nucleic acid concentration.

The presence of proteins in the chromosomes has been demonstrated by Caspersson, who found that the heterochromatic bands could be digested by trypsin, the euchromatic bands also being visibly affected. The results of Barigozzi,[3] who stained salivary gland chromosomes of *Chironomus* with Millon's reagent, indicated the presence of protein along the chromosome, but this author found it impossible to observe much detail in his preparations.

In our experiments we have attempted to investigate the relation between nucleic acid and protein in the structure of the chromosomes by combining the use of histochemical methods for both proteins and nucleic acids with the application of enzymes specific for both substances. The Feulgen-Rossenbeck reaction was used to demonstrate nucleic acids. The ninhydrin reaction proved to be satisfactory as a protein test. Pepsin and trypsin were the proteolytic enzymes employed, and a nucleic-acid splitting enzyme solution was prepared from beef spleen.

Material and Methods.—Salivary glands were dissected from larvae of *Drosophila melanogaster* into insect-Ringer solution.[4] The various treatments were applied to the whole glands, and smear preparations were made just prior to observation. Usually at least ten pairs of glands were carried through a given treatment at a time.

In testing for protein, glands were placed in a drop of 0.2 per cent triketohydrindene hydrate, dissolved in water or Ringer solution. The preparation was heated over a steam bath until the whole gland turned visibly blue. One to two minutes generally sufficed.

The Feulgen-Rossenbeck test was carried out by immersing the glands in 1 *N* HCl at 60°C. for 15 minutes, washing in distilled water and finally

treating with the fuchsin solution for varying lengths of time. Acetocarmine, also used to stain "euchromatin," was applied for 1 to 2 minutes before the glands were spread.

Tryspin solutions were prepared from either commercial (Pfanstiehl 1:110) or crystalline trypsin. In the first case a saturated solution in 0.05 M K_2HPO_4 was filtered and used directly. In tests with crystalline trypsin a 0.1 per cent solution in 0.05 M K_2HPO_4 was employed. In experiments with pepsin a 0.4 per cent solution of HCl was saturated with Merck's pepsin and filtered. Digestion was carried out at 37°C.

A nuclease solution was prepared from beef spleen according to the procedure of van Herwerden.[5] Fresh spleen was ground up, pressed through cheesecloth and shaken with a saturated solution of ammonium sulphate. The material was centrifuged, and the supernatant fluid was dialyzed. The light red solution thus obtained was decanted from the precipitate that formed in the dialysis sac. The solution was brought to pH 7 by means of NaOH and applied to fresh glands at 37°C.

A major difficulty encountered was the failure of the chromosomes to spread after treatment with most of the agents used. This was particularly true in the case of the ninhydrin reaction and the experiments with pepsin. This difficulty does not affect the general conclusions, which concern the reaction of the whole chromosomes, but it would have been most desirable to study the details of banding and to record the results in photographs of isolated chromosomes.

Results. A. The Action of Proteolytic Enzymes.—In so far as the experiments with trypsin were a repetition of those of Caspersson, they confirmed him entirely. Observations were made 3 to 4 hours after the glands were placed in the enzyme solutions. The connective tissue had been almost completely digested so that the individual salivary gland cells tended to separate on handling. Either unstained or treated with acetocarmine, the nuclei appeared to be entirely empty. The chromatin discs observed by Caspersson were lacking since lanthanum ions were not present and the sodium thymonucleate was presumably in solution.

The pepsin solution, on the other hand, did not dissolve any constituent concerned in maintaining the integrity of the chromosome. After pepsin treatment and staining by the Feulgen-Rossenbeck method the chromosomes were still apparent, stained and banded. They were arranged in a rather dense knot, and the volume of the knot and of the individual chromosomes was strikingly reduced as compared with the controls. The controls had been kept in boiled portions of the pepsin solution for the same periods (3 to 30 hours). It is not possible to state whether the loss in volume in the chromosomes was due to digestion of some protein constituent or to dehydration.

Precisely these results were to be expected if the proteins of salivary

gland chromosomes are related to the protamines and histones of the nuclei that have been analyzed by Kossel and others. Both protamines and histones are digested by trypsin. Protamines are not affected by pepsin, while histones are broken down to "histopeptones."[6] The latter seem to be polypeptides rich in arginine, and therefore might be expected to combine with nucleic acids and to remain insoluble under conditions which render protamines insoluble.

These results with pepsin are of particular interest in connection with banded structure. It is commonplace in physiological chemistry that nucleoproteins are only partly digestible by pepsin. But the light bands of the chromosome contain very little nucleic acid. The light regions must, therefore, either contain pepsin-resistant protein, such as protamine or histone, uncombined with nucleic acid, or else the small amount of nucleoprotein present is sufficient to maintain the integrity of these bands. Furthermore, the pepsin-resistant proteins of the light bands must be continuous with the proteins of the euchromatin bands, the continuity being independent of the presence of any pepsin-digestible protein.

B. The Action of Nuclease.—Glands were left in solutions of spleen nuclease for periods up to 48 hours. Controls were placed in boiled samples of the same solution. Clear-cut results were obtained within 22 hours. When glands that had been in boiled nuclease were placed in acetocarmine and observed under low power, the nuclei stood out as bright red spots against a light background within a few minutes. The cytosome scarcely took the stain. Under high power normal banded chromosomes were observed, though they could not be satisfactorily spread for detailed examination.

In glands that had been in active nuclease, and then treated with acetocarmine, the nucleus did not stain at all, even after prolonged immersion in the dye. The cytosome, on the other hand, took the stain quite strongly. Under high power the chromosomes could not be seen at all. The same results were obtained when the Feulgen stain was used. In glands from the boiled nuclease solution the chromosomes stained normally, and the banded structure could be observed. In glands from the active solution, the nucleus did not stain differentially, although in some cases darker areas could be made out that probably were the nuclei. Again the cytosome stained much more strongly than the cytosome in the control glands. Since the treatment of the two groups had been identical throughout, except for the boiling of the nuclease sample for the control, the differences in staining were considered to be attributable to the action of nuclease.

Failure to take nuclear stains did not mean that the chromosomes had been disintegrated by the nuclease. When the nuclease-treated glands were tested by the ninhydrin reaction, the chromosomes appeared very clearly. In this reaction the cytosome takes on a blue color, but the chromosomes stain more intensely. There was no definite evidence of banding

in the ninhydrin-stained chromosomes, although the failure of the chromosomes to spread well made it impossible to draw any positive conclusions. The ninhydrin reaction, as used here, would seem to be preferable to Millon's reaction (Barigozzi), since the latter depends upon phenolic groups which are not plentiful in the nuclear proteins that have been analyzed.[6] It is evident, from these observations, that nuclease has removed the material of the chromosomes that takes so-called nuclear stains without affecting the integrity of the chromosomes themselves.

Discussion.—Previous evidence for the existence of proteins and nucleic acids in chromosomes has already been mentioned. Our experiments have demonstrated that the protein of the chromosomes is at most only partially digested by pepsin, though completely digested by trypsin. This protein, therefore, may be related to the protamines or histones, the traditional nuclear proteins. The loss in volume during pepsin action suggests the presence of a protein that is not involved in the fundamental construction of the chromosome.

These observations with proteolytic enzymes exclude in no way the possibility that the protein is discontinuous, and that regions predominantly protein are held together by chains of nucleic acids. Results of histochemical reactions, such as the Millon reaction (Barigozzi) or the ninhydrin reaction, are not clear enough to establish a continuous protein structure. But the results of the experiments with nuclease seem to be decisive. After treatment with nuclease there is no reaction for nucleic acid in the nucleus, yet the chromosomes are present in their typical form and when stained with ninhydrin cannot be distinguished from controls similarly stained. The structural integrity of even the euchromatic bands does not depend on the presence of intact nucleic acid molecules, though some of the groups left behind after nuclease action may be of importance. The chromosome must have a continuous protein structure, and the nucleic acid must be attached in such a way that it may be split and the pentose may be removed without affecting the continuity of structure.

This conclusion bears on several hypotheses concerning chromosome structure. Wrinch[7] proposed that the chromosomes are composed of a fabric in which polypeptide chains are the warp and polynucleotide molecules, each combining with basic groups of more than one polypeptide chain, the woof. Accepting any of the proposed structures of nucleic acid,[8] it is clear that any splitting of the pentose groups from a polynucleotide will result in breaking of the bonds that hold the nucleotides together. In Wrinch's picture, these bonds hold the polypeptide chains together, and rupturing them should cause a disintegration of the chromosome. However, in the present experiments the pentose was removed (as indicated by a negative Feulgen reaction) without any such disruption of the chromosome. Our data, therefore, do not uphold Wrinch's hypothesis, and the

fibrous structure of the chromosome must be explained by some other mechanism.

The results of the nuclease experiments make it seem improbable that nucleic acid fibres can have anything to do with the cohesion of chromosomes, although Gulick[9] points out that such a structure offers a convenient basis for explaining the genetic phenomenon of inversion. Our results are consistent with the picture based on the birefringence of the euchromatic bands[10] and the light bands,[11] and on the x-ray diffraction of clupein thymonucleate.[12,13] These data lead to a picture of long polypeptide chains and parallel nucleic acid molecules.[14]

In attempting to interpret the results of the experiments with nuclease, it must be remembered that most organ extracts[15] contain at least three specific nucleases: a nucleosidase, a nucleotidase and a polynucleotidase. If the conventional picture of a nucleoprotein as a salt formed by reaction of free amino groups on a polypeptide chain with phosphoric acid residues on the polynucleotide is accepted for the chromosome, the observed result, the removal of the pentose, could only have been effected by the nucleotidase. Conversely, it will be possible to determine how nucleic acids are combined with proteins in chromosomes, by studying the effects of purer specific nucleases by the same methods we have used with the mixed nucleases. Such experiments are being undertaken by us.

The significance of the fact that after nuclease action the cytosome gives a strong Feulgen reaction is not clear, but may conceivably be connected with the variation in the nucleic acid content of chromosomes during mitosis.[2] It would be of interest to determine whether the pyrimidine and purine as well as the pentose components accumulate in the cytosome, and it is planned to test this by ultra-violet photomicrography.

Summary.—1. Salivary gland chromosomes of Drosophila may be completely digested by trypsin, as previously reported by Caspersson.

2. The chromosomes are not digested by pepsin-HCl solutions. This is consistent with the hypothesis that the structural proteins are protamines or histones.

3. After treatment with spleen nuclease, the chromosomes no longer stain with the acetocarmine or Feulgen techniques. Chromosomes treated with boiled nuclease solutions stain normally.

4. In glands treated with active nuclease the cytosome is stained more intensely by these methods than in controls.

5. After treatment with active nuclease, the chromosomes may be stained by the ninhydrin test for protein. The structural framework of the chromosomes, therefore, is not affected by nuclease.

6. It is proposed that the chromosomes possess a continuous protein framework whose integrity is independent of the presence of intact nucleic acid molecules.

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ADJUSTED DEATH RATES

BY CARL R. DOERING AND ALICE L. FORBES

DEPARTMENT OF VITAL STATISTICS, HARVARD SCHOOL OF PUBLIC HEALTH

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The aim of adjusting a death rate is to eliminate the effect of the age distribution and thus make the rates for two districts with different age distributions more fairly comparable. There are several methods of adjustment. We may adopt the following notation: In any age group a for some "standard" population, P_a is the population, D_a is the deaths and $M_a = D_a/P_a$ is the age specific (per capita) death rate. Then the total population $P = \Sigma P_a$, and total deaths $D = \Sigma D_a$, and we may let $F_a = P_a/P$ be the fraction of this standard population in the age group a. Finally $M = D/P$ is the general or crude death rate in the standard. Let small letters replace the capitals when we wish to indicate some particular district, viz.: $m_a = d_a/p_a$ is the age specific death rate, $p = \Sigma p_a$ the population, $d = \Sigma d_a$ the deaths, $f_a = p_a/p$, and the crude death rate of the district is $m = d/p$.

The first and usual method of adjustment[1] is

$$m'_1 = \Sigma m_a F_a, \text{ with } \sigma^2(m'_1) = \sum \frac{m_a(1 - m_a)}{p_a} F_a^2 \qquad (1)$$

as its standard deviation (squared) due to pure chance fluctuations.[2] To compute this requires the knowledge of the age specific death rates, which

practically means of both the death rates d_a and of the population p_a by age, in the district whose death rate is being adjusted. Rates computed in this way may be called "rates adjusted to a standard age distribution of population."

A second method of adjustment[3] is sometimes used as represented by the formula

$$m_2' = \frac{d = \Sigma d_a}{\Sigma M_a p_a} M, \text{ with } \sigma(m_2') = m_2' \frac{\sigma_m}{m}. \qquad (2)$$

This method requires the knowledge of the population distribution in the district but not of the distribution of the deaths. This is a rate adjusted to a standard distribution of specific death rates. However, there seem to be very few cases in which this might be a desirable method, as the distribution of deaths is usually known wherever the population distribution is known, and hence age specific rates could be computed for these places, and the first method or the life table method used.

As vital statistics are reported, however, it often happens that the deaths in a district are available in subdivisions finer than is the population as given by the Census so that it is d_a that is known in detail and not p_a. This suggests a third method of adjustment[4] which would be free of the necessity of knowing p_a, viz.,

$$m_3' = \frac{\Sigma(d_a/M_a)}{p} M, \text{ with } \sigma^2(m_3) = \sum \frac{d_a(1 - m_a)}{M_a^2} \cdot \frac{M^2}{p^2}. \qquad (3)$$

The result for $\sigma^2(m_3')$ requires the knowledge of the age specific death rates m_a in the district though the value of m_3' itself does not; however, the factor $1 - m_a$ is often neglected in computing the (Bernoulli) standard deviation and as its effect is small it can be approximated adequately by using $1 - M_a$ or some other estimate of it whenever it cannot be neglected. This method has a decided advantage over the other methods in that it can be applied by the health officer to places where the age distribution of the population is not known, as in intercensal years, or districts of a large city, or in rural areas.

There is also the life table death rate, which is the reciprocal of the expectation of life at birth $\mathring{e}_o$ and which depends only on the age specific death rates in the district. A short method of calculating $\mathring{e}_o$ and its standard deviation when thus calculated have recently been given; the formulas will not be repeated here.[5]

Finally there is the method of an equivalent average death rate as developed by Yule.[6] In our notation we have

$$m_6' = \frac{\Sigma(h_a m_a)}{\Sigma(h_a M_a)} M, \text{ with } \sigma^2(m_6') = \frac{M^2}{[\Sigma(h_a M_a)]^2} \sum h_a^2 \frac{m_a(1 - m_a)}{p_a} \quad (4)$$

where h_a is the number of years in the age group a, and where the summation is from age zero to an arbitrary age. The latter condition is the inherent difficulty in the method—it cannot cover the entire span of life, and a decision must be made as to where to cut off the death rates. Yule stops at age 65, considers 65–85 separately and ignores the rates over 85 years. As we wished a single figure for comparison with other methods we decided to use ages 0 to 75 in one summation, and ignore the rate 75+, and feel that certain of the results given below justify this decision. A marked advantage of this method is that the ratio of any two adjusted rates is independent of the standard used, and an adjusted rate and its standard deviation due to pure chance on the basis of one standard can be adjusted to that based on another standard merely by multiplying both by an appropriate constant, $\frac{\Sigma(h_a M_{1a})}{\Sigma(h_a M_{2a})} \frac{M_2}{M_1}$.

To show how these various methods behave, there are entered in table 1 the crude death rates, the life table death rates, the equivalent average death rates using ages 0–75 and the adjustments by methods 1, 2 and 3 of the 57 counties of New York State other than New York City. The figures are based on the populations given in the U. S. Census for 1930 (unknown ages omitted and three-year average births substituted for the population under one year) and the deaths reported for 1929–31 inclusive (deaths in state institutions and at unknown ages omitted). The thirteen age groups in which the census populations are given were used.

Two standards were used: first, total New York State population with its age specific death rates, and second, the Wayne County life table population and its death rates. While the latter may seem an unstable standard, its age specific death rates were not irregular, and it was chosen as having the highest expectation of life at birth of any of the counties, and about as high as any country now has. Computations were also made using the Standard Million of England and Wales in 1901, but are not entered here as the adjusted rates were very similar to and only slightly lower than the rates using New York State as a standard. For example, the first method using the Standard Million gives a mean of 10.04, observed standard deviation = 1.25, average chance standard deviation = 0.27 and a Lexian ratio of 4.6. The correlation coefficient of these rates with those using New York State as a standard (same method: first) is 0.997. (The standard deviations due to chance of the second method are so similar to those of the first that it would seem practical to use the simple formula of the second method for the first in ordinary work to test significance of differences between two localities, or the same place at different times.)

TABLE 1

COUNTY	CRUDE DEATH RATE	AGE ADJUSTED DEATH RATE*								
		LIFE TABLE DEATH RATE	STANDARD: NEW YORK STATE			EQUIV. AV. D. R	STANDARD: WAYNE COUNTY LIFE TABLE			
			1ST METHOD	2ND METHOD	3RD METHOD		1ST METHOD	2ND METHOD	3RD METHOD	
Albany	13.41 ± .14	16.94 ± .07	11.81 ± .13	11.84 ± .13	11.50 ± .23	11.85 ± .16	20.18 ± .23	21.90 ± .24	25.51 ± .49	
Allegany	14.90 ± .36	16.33 ± .15	10.18 ± .26	10.44 ± .25	9.77 ± .50	9.47 ± .29	17.81 ± .42	18.14 ± .44	20.78 ± 1.05	
Broome	11.50 ± .16	16.42 ± .08	10.42 ± .14	10.44 ± .15	10.72 ± .28	10.18 ± .18	17.56 ± .26	19.12 ± .27	23.55 ± .60	
Cattaraugus	13.80 ± .25	16.77 ± .11	11.13 ± .21	11.16 ± .20	12.09 ± .42	10.71 ± .24	18.92 ± .34	19.96 ± .36	25.91 ± .88	
Cayuga	14.32 ± .27	16.10 ± .11	10.04 ± .20	10.33 ± .19	10.20 ± .41	9.96 ± .23	17.75 ± .33	18.19 ± .34	21.81 ± .85	
Chautauqua	12.37 ± .18	16.04 ± .08	9.77 ± .15	9.88 ± .14	10.19 ± .29	9.40 ± .17	16.90 ± .24	17.60 ± .26	21.95 ± .61	
Chemung	13.76 ± .25	16.96 ± .12	11.37 ± .21	11.36 ± .20	10.86 ± .38	11.08 ± .25	20.27 ± .34	20.41 ± .36	23.99 ± .81	
Chenango	16.95 ± .40	16.77 ± .18	10.98 ± .29	10.98 ± .26	11.70 ± .60	10.77 ± .32	18.58 ± .43	18.84 ± .44	25.03 ± 1.25	
Clinton	13.85 ± .31	17.23 ± .15	11.57 ± .26	11.72 ± .26	11.79 ± .52	11.04 ± .32	19.15 ± .44	20.83 ± .47	25.55 ± 1.10	
Columbia	15.08 ± .34	16.49 ± .15	10.68 ± .26	10.69 ± .24	11.55 ± .54	10.00 ± .29	18.22 ± .41	18.74 ± .43	24.82 ± 1.13	
Cortland	16.16 ± .41	16.96 ± .18	11.60 ± .31	11.75 ± .30	11.73 ± .61	10.91 ± .35	20.09 ± .50	20.71 ± .52	25.31 ± 1.29	
Delaware	14.65 ± .34	16.36 ± .15	10.21 ± .25	10.39 ± .24	10.09 ± .49	9.63 ± .28	17.73 ± .40	18.17 ± .42	21.76 ± 1.03	
Dutchess	14.29 ± .21	17.07 ± .11	11.55 ± .18	10.96 ± .16	13.70 ± .36	10.49 ± .19	17.89 ± .27	19.66 ± .29	30.37 ± .76	
Erie	11.78 ± .07	17.16 ± .04	12.05 ± .07	12.07 ± .07	12.47 ± .13	11.93 ± .09	19.94 ± .13	22.81 ± .14	27.67 ± .28	
Essex	16.15 ± .39	18.42 ± .20	14.10 ± .35	13.36 ± .33	17.93 ± .75	12.35 ± .38	21.31 ± .53	23.90 ± .58	38.10 ± 1.53	
Franklin	16.67 ± .35	19.38 ± .20	15.40 ± .32	14.42 ± .30	20.58 ± .68	13.06 ± .33	22.19 ± .47	25.88 ± .54	44.51 ± 1.43	
Fulton	14.46 ± .32	16.16 ± .13	10.20 ± .24	10.45 ± .23	8.91 ± .39	9.96 ± .27	18.10 ± .39	18.60 ± .41	19.65 ± .84	
Genesee	13.10 ± .31	16.40 ± .14	10.40 ± .25	10.53 ± .25	10.36 ± .49	9.98 ± .29	17.97 ± .43	18.79 ± .45	22.45 ± 1.04	
Greene	15.39 ± .44	16.20 ± .19	9.98 ± .32	10.01 ± .29	10.75 ± .65	9.05 ± .33	17.22 ± .48	17.45 ± .50	22.86 ± 1.34	
Hamilton	11.36 ± .98	16.59 ± .55	10.31 ± .91	9.71 ± .84	14.28 ± 2.05	8.40 ± .90	16.10 ± 1.44	17.82 ± 1.54	30.44 ± 4.23	
Herkimer	12.70 ± .26	16.32 ± .12	10.20 ± .21	10.15 ± .20	10.69 ± .42	9.74 ± .25	17.22 ± .34	18.11 ± .36	23.30 ± .89	
Jefferson	14.57 ± .24	16.45 ± .10	10.51 ± .18	10.68 ± .18	10.89 ± .37	10.18 ± .21	18.19 ± .29	18.80 ± .31	23.43 ± .77	
Lewis	12.72 ± .42	15.52 ± .17	8.50 ± .30	9.09 ± .30	7.43 ± .56	8.14 ± .35	15.68 ± .51	15.84 ± .53	15.89 ± 1.18	
Livingston	11.31 ± .32	15.25 ± .13	8.10 ± .24	8.55 ± .24	6.35 ± .39	7.70 ± .28	15.00 ± .41	15.05 ± .42	13.64 ± .83	
Madison	15.30 ± .36	16.17 ± .14	10.05 ± .25	10.40 ± .24	9.88 ± .51	9.73 ± .28	17.77 ± .40	18.05 ± .42	21.05 ± 1.06	
Monroe	10.66 ± .09	16.06 ± .04	9.87 ± .08	9.91 ± .08	9.23 ± .15	9.98 ± .11	17.30 ± .15	18.42 ± .16	20.27 ± .32	
Montgomery	12.76 ± .26	16.51 ± .13	10.65 ± .22	10.61 ± .22	10.61 ± .41	10.23 ± .26	18.02 ± .38	19.15 ± .40	23.02 ± .87	
Nassau	8.76 ± .10	15.95 ± .05	9.77 ± .11	9.65 ± .11	9.22 ± .18	9.84 ± .15	17.37 ± .22	18.57 ± .21	20.26 ± .39	
Niagara	10.82 ± .15	16.51 ± .08	10.73 ± .15	10.74 ± .15	11.24 ± .29	10.30 ± .19	17.97 ± .28	20.00 ± .29	24.71 ± .61	
Oneida	12.79 ± .15	16.68 ± .07	10.96 ± .13	11.00 ± .13	10.89 ± .24	10.68 ± .15	18.76 ± .22	19.97 ± .23	23.95 ± .51	
Onondaga	11.65 ± .11	16.31 ± .05	10.46 ± .10	10.53 ± .10	9.84 ± .18	10.39 ± .13	18.22 ± .19	21.01 ± .21	21.72 ± .39	
Ontario	15.71 ± .31	16.77 ± .13	11.39 ± .23	11.58 ± .23	11.02 ± .45	11.06 ± .27	19.77 ± .38	20.48 ± .40	23.80 ± .94	
Orange	13.75 ± .19	16.80 ± .09	11.20 ± .15	11.07 ± .15	12.57 ± .32	10.66 ± .18	18.70 ± .26	20.05 ± .27	27.37 ± .68	
Orleans	12.95 ± .38	15.59 ± .16	8.71 ± .28	9.09 ± .27	7.88 ± .51	8.53 ± .32	15.92 ± .45	15.81 ± .47	16.64 ± 1.05	
Oswego	14.51 ± .26	16.48 ± .11	10.48 ± .20	10.68 ± .19	9.88 ± .37	10.30 ± .23	18.25 ± .32	18.78 ± .34	21.21 ± .79	
Otsego	17.13 ± .35	16.66 ± .15	10.87 ± .24	11.22 ± .23	10.32 ± .46	10.55 ± .27	19.11 ± .37	19.39 ± .39	22.01 ± .96	
Putnam	12.27 ± .54	16.06 ± .26	9.45 ± .44	9.40 ± .42	9.93 ± .87	8.56 ± .48	16.08 ± .70	16.69 ± .74	21.17 ± 1.79	
Rensselaer	15.13 ± .20	17.16 ± .10	12.04 ± .16	12.02 ± .16	12.00 ± .31	12.04 ± .19	20.36 ± .28	21.82 ± .29	26.44 ± .66	
Rockland	11.26 ± .25	16.51 ± .13	10.57 ± .23	10.55 ± .23	11.02 ± .46	10.23 ± .28	17.74 ± .41	19.33 ± .43	23.92 ± .96	
St. Lawrence	14.04 ± .23	16.55 ± .10	10.09 ± .17	10.24 ± .16	10.07 ± .34	9.47 ± .19	17.01 ± .27	17.88 ± .29	21.64 ± .72	

Saratoga	13.73 ± .27	16.31 ± .12	10.45 ± .21	10.56 ± .21	10.23 ± .40	10.20 ± .24	18.13 ± .35	18.87 ± .37	22.14 ± .84
Schenectady	10.85 ± .17	16.41 ± .08	10.77 ± .16	10.77 ± .17	10.29 ± .29	10.65 ± .21	18.89 ± .32	20.35 ± .32	22.61 ± .61
Schoharie	16.70 ± .53	15.87 ± .20	9.25 ± .34	9.84 ± .31	8.51 ± .63	8.87 ± .36	16.70 ± .51	16.72 ± .53	17.83 ± 1.32
Schuyler	15.08 ± .62	16.25 ± .27	9.86 ± .45	9.99 ± .41	10.31 ± .94	9.19 ± .49	16.94 ± .68	17.22 ± .71	21.48 ± 1.91
Seneca	13.42 ± .42	15.46 ± .18	8.30 ± .29	8.34 ± .26	8.00 ± .53	7.52 ± .30	14.45 ± .44	14.48 ± .45	16.95 ± 1.11
Steuben	15.83 ± .25	17.10 ± .11	11.75 ± .19	11.72 ± .19	11.65 ± .36	11.22 ± .22	19.78 ± .31	20.69 ± .33	25.36 ± .77
Suffolk	11.87 ± .16	16.26 ± .07	10.10 ± .13	10.07 ± .13	11.18 ± .28	9.76 ± .16	17.15 ± .23	18.35 ± .24	24.17 ± .58
Sullivan	16.06 ± .39	18.36 ± .22	13.99 ± .35	12.60 ± .30	18.81 ± .73	11.55 ± .33	20.39 ± .50	22.71 ± .55	40.21 ± 1.52
Tioga	14.71 ± .44	15.76 ± .18	9.11 ± .30	9.51 ± .28	8.80 ± .60	8.71 ± .33	16.23 ± .47	16.34 ± .48	18.79 ± 1.25
Tompkins	14.16 ± .33	16.11 ± .14	10.20 ± .25	10.43 ± .25	10.33 ± .50	10.12 ± .30	17.87 ± .42	18.35 ± .43	22.33 ± 1.05
Ulster	15.90 ± .25	17.20 ± .12	11.94 ± .20	11.77 ± .19	12.85 ± .41	11.77 ± .22	19.79 ± .32	20.96 ± .34	27.67 ± .85
Warren	15.67 ± .39	16.91 ± .17	11.71 ± .30	11.60 ± .29	12.47 ± .60	11.46 ± .35	19.58 ± .48	20.64 ± .51	27.10 ± 1.27
Washington	13.01 ± .30	16.01 ± .13	9.58 ± .23	9.72 ± .23	9.12 ± .44	9.43 ± .27	16.59 ± .38	17.17 ± .40	19.69 ± .93
Wayne	12.75 ± .29	15.14 ± .11	8.05 ± .20	8.73 ± .20	7.29 ± .38	7.85 ± .23	15.14 ± .33	15.14 ± .34	15.14 ± .77
Westchester	9.59 ± .08	16.17 ± .04	10.21 ± .08	10.17 ± .08	9.84 ± .14	10.31 ± .11	17.66 ± .16	19.36 ± .16	21.62 ± .30
Wyoming	14.14 ± .40	15.83 ± .16	9.34 ± .29	9.76 ± .28	9.12 ± .59	9.18 ± .33	16.71 ± .46	16.98 ± .48	19.42 ± 1.22
Yates	16.21 ± .56	15.95 ± .21	9.59 ± .37	10.11 ± .35	10.38 ± .81	8.98 ± .41	17.21 ± .58	17.34 ± .60	21.79 ± 1.67
Mean	13.76 .34	16.49 .16	10.57 .27	10.62 .26	10.90 .55	10.09 .30	18.03 .43	18.99 .45	23.54 1.15
Observed σ	1.92	.72	1.34	1.09	2.46	1.16	1.56	2.14	5.33
Lexian Ratio	5.6	4.5	5.0	4.3	4.5	3.9	3.6	4.7	4.7
6 Groups									
(Mean)	13.76	16.38	10.80	10.90	11.03				
(Observed σ)	1.92	.73	1.33	1.11	2.37				
53 Counties									
Mean	*13.61 .34*	*16.36 .16*	*10.33 .27*	*10.45 .25*	*10.38 .54*	*9.96 .30*	*17.84 .43*	*18.68 .45*	*22.43 1.13*
Observed σ	*1.88*	*.49*	*.98*	*.87*	*1.48*	*1.06*	*1.39*	*1.79*	*3.31*
Lexian Ratio	*5.5*	*3.1*	*3.7*	*3.4*	*2.7*	*3.6*	*3.2*	*4.0*	*2.9*
6 Groups									
(Mean)	*13.61*	*16.25*	*10.56*	*10.73*	*10.53*				
(Observed σ)	*1.88*	*.54*	*.99*	*.90*	*1.43*				

*The results by the method of equivalent average death rates are given only for New York State as a standard. If those using Wayne County life table as a standard are desired, one needs merely to multiply each death rate with its σ by 1.9280 and hence the mean, σ, and average σ by this same figure. The Lexian ratio and all correlations will remain unchanged.

The figures whose side headings are in parentheses at the foot of the table are for the case when the 6 age groups of the Census Bureau are used. The correlations of the values obtained for the 57 counties from the 13 groups with those from 6 groups are .984, .991, .985, .997 for the life table and for methods 1, 2 and 3, respectively, using New York State as a standard. With the coarser grouping the three adjusted death rates are higher on the average and the detailed tables show they are individually higher in most cases, whereas the life table death rate is lower on the average and usually in the individual counties.

The figures in italics are the results if only 53 counties are used, omitting Dutchess, Essex, Franklin and Sullivan Counties. These four are "tuberculosis counties" and have crude tuberculosis death rates ranging from 19.5 per 10,000 population for Dutchess County to 41.5 for Franklin County. Since all the other counties had rates of 10.2 or lower, it seemed desirable to compare the results when the unusual counties were omitted with when they were included.

There are four counties which have large tuberculosis death rates; the most striking effect of omitting these counties is the reduction of the observed standard deviation, and consequently of the Lexian ratio, of the adjusted rates.

A desirable feature in an adjusted rate is that if two districts have identical age specific death rates, their adjusted rates should be equal. This is true of the life table death rate, of the first method, and of the equivalent average death rate. However, the second method was only 5% out and the third method less than 2% out when Nassau County was compared with an imaginary county having the same age specific death rates but the population distribution of Seneca County. Another desirable feature is to have the adjusted rate in a given locality doubled if the age specific rates are doubled. This is true in all methods except the life table death rate, which is only about 25% greater when the mortalities are doubled.

TABLE 2

CORRELATION COEFFICIENTS*

		CRUDE	LIFE TABLE	NEW YORK STATE 1ST	NEW YORK STATE 2ND	NEW YORK STATE 3RD	NEW YORK STATE EQ. AV.	WAYNE LIFE TABLE 1ST	WAYNE LIFE TABLE 2ND	WAYNE LIFE TABLE 3RD	LOG. POPULATION
	Crude	.984	.408	.387	.454	.357	.299	.414	.225	.326	−.478
		.983	*.274*	*.243*	*.349*	*.183*	*.182*	*.314*	*.053*	*.130*	*−.476*
	Life Table		.975	.987	.955	.945	.868	.884	.920	.952	.110
			.947	*.978*	*.947*	*.874*	*.894*	*.902*	*.903*	*.893*	*.279*
New York State	1st			.979	.979	.924	.923	.928	.959	.939	.191
				.962	*.978*	*.850*	*.953*	*.947*	*.956*	*.884*	*.372*
	2nd				.972	.849	.952	.972	.963	.862	.215
					.957	*.765*	*.959*	*.980*	*.943*	*.792*	*.363*
	3rd					.975	.732	.745	.820	.998	−.012
						.930	*.699*	*.693*	*.745*	*.995*	*.109*
	Eq. Av.						.967	.970	.963	.759	.435
							.960	*.965*	*.962*	*.742*	*.550*
Wayne Life Table	1st							.961	.952	.764	.297
								.951	*.941*	*.727*	*.412*
	2nd								.977	.844	.389
									.968	*.791*	*.558*
	3rd									.977	.038
										.940	*.184*

* The coefficients in italics are based on 53 counties, omitting the 4 tuberculosis counties. The self correlations in the main diagonal are computed from the standard errors of pure chance by the well-known formula $r_{11}^2 = 1 - \sigma_e^2/\sigma_c^2$ where σ_e^2 is the average of the square of the error and σ_c^2 is the variance of the quantity which is to be self correlated; they indicate the correlation between two determinations of the same thing when only chance variations occur.

The intercorrelations of the crude death rates, their various adjustments and the logarithm of the population of the county are entered in table 2. The negative correlation between the crude death rate and log p is a reflection of the fact that the smaller counties have on the whole an older population, the correlation between log p and the average age of the population being -0.581.

Of the first three methods, which are influenced by the standard chosen, the third method is least affected by a change in standard, and also has the lowest correlation with the logarithm of the population. The method of equivalent average death rates weights the older age groups relatively heavily, so in comparing it with the first method, it seems fairer to use those values obtained by using the life table population as a standard. The relatively high correlation of the generally approved first method (with the life table standard) and the equivalent average death rate seems to indicate that we have not introduced too gross an error in the latter by lumping together all age groups up to age 75 and ignoring those 75 and over. The life table death rate weights the earlier age groups relatively heavily, as may really be desirable if the death rate is to be taken as a sanitary index.

On the whole, it seems that the second method is of very little use, and of doubtful value; the first method is desirable when the data are available if the standard is carefully chosen; the equivalent average method requires the same data, but is independent of the standard, although it weights the older ages more than is generally desired; the third method has great utility where the others cannot be applied because of insufficient knowledge of the population distribution; and the life table death rate is valuable in comparing places with widely different population distributions since no standard need be selected. The choice of a suitable method in any particular instance will depend on the data available, the emphasis desired and the similarity of the places to be compared.

[1] This method is that of Newsholme, *Vital Statistics*, 1924, p. 219; of Whipple, *Vital Statistics*, 1923, p. 291; of Pearl, *Medical Biometry and Statistics*, 1930, p. 269; of Woods and Russell, *Introduction to Medical Statistics*, 1936, p. 44.

[2] The term F_a is not subject to fluctuation, but only m_a; the regular formula due to Bernoulli is used for $\sigma(m_a)$.

[3] Newsholme, loc. cit., p. 222; Whipple, loc. cit., p. 292 (with, however, an error whereby a fraction is inverted); Pearl, loc. cit., p. 269; Woods and Russell, loc. cit., p. 51.

[4] Logically there is a fourth method $m'_4 = \dfrac{P}{\Sigma D_a/m_a}\ M$, but this seems to possess no advantages, as m_a implies the knowledge of both deaths and populations in the district by age, and if these were known, we should certainly use one of the other three formulas.

[5] Doering, C. R., and Forbes, A. L., *Proc. Nat. Acad. Sci.*, **24**, 400–405 (1938); and Wilson, E. B., *Jour. Am. Stat. Assoc.*, **33**, 705–708 (1938). Here $m'_5 = \dfrac{1}{\overset{\circ}{e}_0}$, $\sigma(m'_5) = \dfrac{\overset{\circ}{\sigma_{e_0}}}{\overset{\circ}{e}_0}\ m'_5$.

[6] Yule, G. U., "On Some Points Relating to Vital Statistics, More Especially Statistics of Occupational Mortality," *Jour. Roy. Stat. Soc.*, **97**, 15ff (1934).

THE GRAVITATIONAL MOTION OF A PAIR OF RIGID BODIES

BY THEODORE EUGENE STERNE

HARVARD COLLEGE OBSERVATORY

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1. In a paper[1] on eclipsing binary stars, Kurt Walter studied the dynamical nature of the gravitational motion of a pair of rigid bodies. The bodies are considered to be ellipsoids, with their shortest axes normal to the orbital plane. The long axes of the ellipsoids librate about the line of centers in the plane of the orbit, causing the motion to be not accurately elliptical. Walter found, for small librations, that when the mean distance and the physical parameters characterizing the bodies have certain values, the line of apsides regresses; for other values it advances; and for still others, the motion is unstable. Walter's solution was adversely criticized on dynamical grounds by Kopal,[2] who obtained a solution that differs from Walter's, and according to which the line of apsides always advances when the librations are small. More recently, Kopal's treatment has in turn been adversely criticized, on dynamical grounds, by Cowling.[3] It is, actually, most unlikely that the solution of Walter's problem can be applicable to binary stars. Walter[4] now agrees with Cowling and the author, who have developed[3,5] the theory of the motion of binary stars having fluid components, that the figures of gaseous (and therefore of fluid) components must change from instant to instant under the action of the changing tidal forces.*

The solution of the rigid-body problem proposed by Walter, even if it is not applicable to binary stars, must nevertheless be definite. There can be no real uncertainty about it, and since the problem is interesting from the point of view of celestial mechanics, Professor E. F. Freundlich has suggested to me that it might be desirable to try to clear up whatever apparent uncertainty there may be. Professor H. N. Russell has pointed out that Walter's problem is of a type that can be handled by the theory of vibrations about steady motion. In this note, I shall show briefly that the

* In the fluid-body problem, there are no librations in the "rigid-body" sense. There are internal modes of free vibration of the bodies themselves, but unless the bodies are nearly in contact such internal modes can be shown to have periods that are short in comparison with the orbital period, and the free oscillations of the internal modes are not excited. Forced vibrations of the internal modes exist, however, corresponding to partial tides in the fluid bodies. The geometrical figures, given by the resultants of such partial tides, are always periodic in the orbital period. When the free internal periods are short compared with the orbital period, the line of apsides advances. For a discussion of the tidal problem, see Sterne, references 5 and 6; and for applications to some eclipsing binary stars, to determine concentrations of density, see Sterne, references 6 and 7.

method of that theory, applied to Walter's problem, leads precisely to Walter's solution.

2. In order to obtain results directly comparable to Walter's, we shall use his notation to as great an extent as possible. We shall not, however, require the two bodies to be elliptical or homogeneous. Let their masses be m_1 and m_2; let the distance between centers of mass be r; let l be the longitude of the line of centers; let a_i, b_i and c_i denote the principal axes of inertia of the bodies; and let k_a, k_b, k_c and k'_a, k'_b, k'_c be the corresponding radii of gyration of bodies 1 and 2, respectively, k_c and k'_c being largest in each case. The c-axes are normal to the orbital plane. Let v_1 be the angle, measured in the sense of the orbital motion, from the line of centers to the axis a_1 in the orbital plane; and let v_2 be the corresponding angle for body 2. We are interested in small vibrations of the system about a state of steady motion in which r is equal to a, some constant, and in which the angles of libration, v_1 and v_2, have permanent zero values. Like Walter, we are not interested in the possible librations of the directions of the c-axes which, if they initially maintain directions normal to the orbital plane, will remain normal to it. We must, however, take into account the changes in r, v_1 and v_2. It is known** that the gravitational potential due to body 1, at the center of mass of body 2, is

$$\frac{k^2 m_1}{r} + \frac{k^2 m_1}{2r^3}(k_a^2 + k_b^2 + k_c^2 - 3k_r^2)$$

plus terms involving higher negative powers of r, where k_r is the radius of gyration of body 1 about the line of centers, and k is the Gaussian constant of gravitation. It follows from the properties of momental ellipsoids that

$$k_r^2 = k_a^2 + (k_b^2 - k_a^2) \sin^2 v_1$$

exactly, and thus one may write down the kinetic and potential energies of the system in the forms

$$T = \frac{1}{2}(\dot{r}^2 + r^2\dot{l}^2) + \frac{1}{2}T_1(\dot{l} + \dot{v}_1)^2 + \frac{1}{2}T_2(\dot{l} + \dot{v}_2)^2,$$

$$V = -\frac{\alpha}{r} - \frac{\alpha a^2}{3r^3}\eta + \frac{1}{r^3}(\gamma_1 \sin^2 v_1 + \gamma_2 \sin^2 v_2).$$

Here T and V are the kinetic and potential energies, each multiplied by the constant $(m_1 + m_2)/m_1 m_2$, and

$$T_1 = \frac{m_1 + m_2}{m_2} k_c^2, \qquad T_2 = \frac{m_1 + m_2}{m_1} k'^2_c, \qquad \alpha = k^2(m_1 + m_2),$$

** See, for example, Plummer, reference 8.

$$\eta = \frac{3}{2a^2}(k_b^2 + k_c^2 - 2k_a^2 + k'^2_b + k'^2_c - 2k'^2_a),$$

$$\gamma_1 = \frac{3\alpha}{2}(k_b^2 - k_a^2), \qquad \gamma_2 = \frac{3\alpha}{2}(k'^2_b - k'^2_a).$$

The inclusion of the constant factors in T and V is obviously without effect upon the Lagrangian equations of motion. Corresponding to the ignorable coördinate l, there exists the integral

$$(r^2 + T_1 + T_2)\dot{l} + T_1\dot{v}_1 + T_2\dot{v}_2 = h, \tag{1}$$

a constant. The modified kinetic potential, after ignoration of l, is therefore***

$$\left.\begin{aligned} R = T - V - h\dot{l} \\ = \frac{1}{2}\dot{r}^2 - \frac{1}{2}\frac{(T_1\dot{v}_1 + T_2\dot{v}_2)^2}{r^2 + T_1 + T_2} + \frac{1}{2}(T_1\dot{v}_1^2 + T_2\dot{v}_2^2) \\ - \frac{h^2}{2(r^2 + T_1 + T_2)} + \frac{h(T_1\dot{v}_1 + T_2\dot{v}_2)}{r^2 + T_1 + T_2} + \frac{\alpha}{r} \\ + \frac{\alpha a^2}{3r^3}\eta - \frac{1}{r^3}(\gamma_1 \sin^2 v_1 + \gamma_2 \sin^2 v_2) \end{aligned}\right\} \tag{2}$$

and the equations of motion are

$$\frac{d}{dt}\left(\frac{\partial R}{\partial \dot{q}_j}\right) - \frac{\partial R}{\partial q_j} = 0; \quad j = 1, 2, 3, \tag{3}$$

where $q_1 = r$, $q_2 = v_1$, $q_3 = v_2$.

In the steady motion $(\partial R/\partial q_j) = 0$; $j = 1, 2, 3$. The first condition gives

$$h^2 = \alpha a(1 + \eta)\delta^2$$

where $\delta = 1 + \tau_1 + \tau_2$, $\tau_1 = T_1/a^2$, $\tau_2 = T_2/a^2$; and the other two conditions are automatically satisfied. It follows from (1) that the angular velocity in the steady motion, and therefore, very nearly, the mean orbital angular velocity when the librations are small, is given by n where

$$n^2 = (\alpha/a^3)(1 + \eta).$$

We set $r = a + x$, and expand R by Taylor's theorem in ascending powers of the coördinates x, v_1, v_2, ignoring terms in the expansion that are above the second degree in x, v_1, v_2, $\dot{x}$, $\dot{v}_1$, $\dot{v}_2$. Since the equations of motion (3) are satisfied by permanent zero values of the coördinates, there are no

*** The reader is referred to Whittaker, reference 9, for the dynamical principles employed.

terms linear in the coördinates and independent of the velocities. Terms linear in the velocities and independent of the coördinates disappear from the equations of motion and can be omitted. We thus find

$$R = \frac{1}{2}\dot{x}^2 - \frac{a^2}{2\delta}\left[(\tau_1^2 - \tau_1\delta)\dot{v}_1^2 + 2\tau_1\tau_2\dot{v}_1\dot{v}_2 + (\tau_2^2 - \tau_2\delta)\dot{v}_2^2\right]$$
$$+ \frac{\alpha}{2a^2\delta}(1+\eta)\left[-1 + 3(\tau_1+\tau_2) + 2\frac{\delta\eta}{1+\eta}\right]x^2$$
$$- \frac{1}{a^3}(\gamma_1 v_1^2 + \gamma_2 v_2^2) - \frac{2}{\delta}\left[\frac{\alpha}{a}(1+\eta)\right]^{1/2}(\tau_1\dot{v}_1 + \tau_2\dot{v}_2)x. \qquad (4)$$

By the theory of vibrations about steady motion, any solution of the equations of motion is known either to be of the form

$$x = x_0 e^{sit},\ v_1 = v_0 e^{sit},\ v_2 = v'_0 e^{sit},$$

or to be a linear combination of such forms. It is convenient to introduce the ratio $\lambda = s/n$. Then the equations of motion are found from (4) to be

$$\left.\begin{aligned}
&\left[\delta\lambda^2 - 1 + 3(\tau_1+\tau_2) + 2\frac{\delta\eta}{1+\eta}\right]x - 2a\tau_1\lambda i v_1 - 2a\tau_2\lambda i v_2 = 0\\
&\frac{2}{a}\lambda i x + [(1+\tau_2)\lambda^2 - x_1]v_1 - \tau_2\lambda^2 v_2 = 0\\
&\frac{2}{a}\lambda i x - \tau_1\lambda^2 v_1 + [(1+\tau_1)\lambda^2 - x_2]v_2 = 0
\end{aligned}\right\} \qquad (5)$$

where

$$x_1 = \frac{2\gamma_1\delta}{\alpha T_1(1+\eta)},\quad x_2 = \frac{2\gamma_2\delta}{\alpha T_2(1+\eta)}.$$

These equations are consistent only if λ has such a value that the determinant, Δ, of the coefficients vanishes; that is to say, only if $\lambda = \pm y^{1/2}$ where y satisfies the cubic equation

$$\left\{\delta y - 1 + 3(\tau_1+\tau_2) + 2\frac{\delta\eta}{1+\eta}\right\}\{\delta y^2 - [x_1(1+\tau_1)$$
$$+ x_2(1+\tau_2)]y + x_1x_2\} - 4\tau_1 y(\delta y - x_2) - 4\tau_2 y(\delta y - x_1) = 0, \qquad (6)$$

and then the constants x_0, v_0, v'_0 are proportional to the cofactors of the terms in any row of Δ.

If all three roots of the cubic are real and positive, the steady motion is stable; and to each of the roots there corresponds a normal mode of vibration, about the steady motion, having a period $2\pi/|s|$ and of the form

$$x = A \cos st, \; v_1 = B \sin st, \; v_2 = C \sin st, \qquad (7)$$

where the coefficients A, B, C are any real numbers such that A, $-Bi$, $-Ci$ are proportional to the cofactors of the terms in the top row of the determinant Δ. In (7), s is to be regarded as positive; and λ is to be regarded as positive in the evaluation of the cofactors.† The time t may be measured from an arbitrarily chosen epoch. There will be in general three modes of the form (7), corresponding to the three roots of equation (6), and the most general solution is any linear combination of them. One of the modes corresponds to the principal elliptic term in the motion, and to a root of (6), λ_a say, that is close to unity. The mode has a period, which may be called the "radial" period, that is close to the orbital period; and the nature of the mode may be most clearly visualized by considering the variations of the orbital angular velocity during a radial period. At pericenter, the orbital angular velocity is largest; at apocenter, smallest. The bodies rotate less rapidly than the orbital motion at pericenter, and more rapidly at apocenter. The angles of libration, v_1 and v_2, are zero at pericenter, and are decreasing. They are negative between pericenter and apocenter, zero at apocenter and positive between apocenter and pericenter. It is this mode which is responsible for the motion of the line of apsides. If λ exceeds unity, the radial period is shorter than the orbital, and the line of apsides regresses; if λ is less than unity, the line advances. The ratio of the rate of advance to the mean motion n in the orbit is $1 - \lambda$, of which a negative sign corresponds to regression. The other two modes correspond to librations v_1 and v_2 that are more purely gravitational in nature; in one of them these librations have identical signs, in the other opposite signs; they appear always to have periods long compared with the orbital period, and to involve oscillations in r that are relatively smaller, in comparison with the amplitudes of the v's, than in the case of the first mode. If the two bodies are identical, it is seen from the equations (5) that the long-period mode for which the v's have opposite signs involves no changes at all in r. The two long-period modes are always possible when the steady motion is stable, but they have nothing directly to do with the apsidal motion, and even in an eccentric orbit they need not be excited—that is to say, their A's, B's and C's may all be zero. If they are excited, they merely superimpose their own small displacements upon those of the first mode which, by its nature, is present whenever the orbit is eccentric, and which is, in fact, the orbital eccentricity.

Unless all three roots of the cubic are real and positive, the state of steady motion is unstable. There is then at least one "mode" that contains an

† If a negative value of s is used, then a negative value of λ must also be used, in the cofactors; the signs of B and C are thereby changed, and (7) is left unaltered. The solution (7) has been obtained, of course, by adding together two solutions, involving $\exp(sit)$ and $\exp(-sit)$ respectively.

exponential term involving a real and positive multiple of the time, and which is not, therefore, a vibration.

Our equation (6) is exactly Walter's equation[1] (11), Walter's equations (9) constitute the linear combination of normal modes referred to previously, and since Walter's "ρ" is our x/a his equations (10) express the ratios between A, B and C in terms, precisely, of the cofactors of the top row of our determinant Δ. In deriving his equations Walter considered homogeneous ellipsoids, whereas we have considered inhomogeneous irregular bodies. We have taken care, however, to keep identical the meanings of identical symbols in the special case when the bodies are homogeneous ellipsoids. Thus the solution obtained here is exactly equivalent to the solution given by Walter.

It is a simple matter, in any numerically specified example, to solve equation (6) and thus to determine whether the steady motion is stable or unstable, and, if the steady motion is stable, whether the apsidal motion is one of advance or regression. A numerical instance of an unstable case has been given by Walter.†† An example of a stable case, with regression, is furnished by two homogeneous rigid bodies of equal masses, with mean radii (averaged over the surfaces) of 0.3, and having the same ellipsoidal figures that they would have if, instead of being rigid, they were fluid, and were in equilibrium under the action of the tidal and centrifugal forces and their own gravitation.††† I am indebted to Dr. Kopal for suggesting a numerical example in which there is found to be stability and an apsidal precession. In this example, the masses of the homogeneous rigid ellipsoidal bodies are $m_1 = 10$ and $m_2 = 1$; the mean radii are 0.15 and 0.05, respectively. The bodies 1 and 2 have for their semi-axes the equilibrium values 0.15036, 0.15017, 0.14947 and 0.05018, 0.04995, 0.04986, respectively, in the directions of the principal axes a_i, b_i, c_i. It is found that $\lambda_a = 0.999983$. In both examples, $a = 1$.

3. *Summary.*—Walter has studied the nature of the nearly elliptical motion of a pair of rigid bodies, whose small librations in the orbital plane are taken into account. Walter's solution of this problem, although inapplicable probably to real binary stars, is nevertheless of dynamical interest. His solution, which has been adversely criticized on dynamical grounds, is here completely confirmed. Under appropriate circumstances instability, apsidal precession or apsidal regression may each occur.

†† Reference 1, page 8.

††† The formulae for computing such ellipsoidal figures may be found in Sterne, reference 5, page 460.

[1] Walter, *Veröff. der Universitäts-Sternwarte*, Königsberg, 3 (1933).
[2] Kopal, *M. N.*, **98**, 448 (1938).
[3] Cowling, *M. N.*, **98**, 734 (1938).
[4] Walter, *Zs. für Astrophysik*, **17**, 320 (1939).

[5] Sterne, *M. N.*, **99**, 451 (1939).
[6] Sterne, *M. N.*, **99**, 662 (1939).
[7] Sterne, *M. N.*, **99**, 670 (1939).
[8] Plummer, *Dynamical Astronomy*, Chapter II.
[9] Whittaker, *Analytical Dynamics*.

A THEORY OF LENGTH AND ITS APPLICATIONS TO THE CALCULUS OF VARIATIONS

BY KARL MENGER

UNIVERSITY OF NOTRE DAME

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1. *Variational Distance and Geometric Distance.*—Let S be a limit class, i.e., a set for whose points convergence is defined. By a curve we mean a continuous mapping of a closed interval of real numbers into S. On the basis of the definition of convergence for points we can define convergence for sequences of curves.

Let a distance be associated with each ordered pair of elements of S. Under which conditions can we say that each curve in S has a length derived from this distance, and that this length is a lower semicontinuous (l.s.c.) functional of the curves? By length of a curve C derived from a distance we mean a finite or infinite number to which the lengths of all sufficiently dense subpolygons of C formed by means of the distance come as close as we please.

It is convenient to assume[1] that the definition of convergence in S is based on a distance $\delta(p, q)$ associated with the pairs of points of S. But this distance plays merely an auxiliary role, and is by no means necessarily the same as the distance from which we derive the length of curves in whose existence and semicontinuity we are interested. We call $\delta(p, q)$ the *geometric* or *δ-distance*, and denote by $\delta_1(p, q)$ the distance from which we derive the length. The latter will be called *variational* or *δ_1-distance*. In the applications to problems of the calculus of variations the geometric distance by which we determine the neighborhood of points and curves will be the ordinary Euclidean distance which when used in defining the length of a curve yields its ordinary Euclidean length. The variational distance, however, when used in defining the length of a curve C yields the integral along C of the integrand of the variational problem.

2. *The Semicontinuity of the Variational Length.*—We make the following assumptions:

I. δ is *normal:* $\delta(p, p) = 0$ for each p.

II. δ is *uniformly continuous:* For each $\epsilon > 0$ there exists a $\delta(\epsilon) > 0$ such that $|\delta(p, p')| + |\delta(q, q')| < \delta(\epsilon)$ implies $|\delta(p, q) - \delta(p', q')| < \epsilon$.

III_1. δ_1 is an *upper substitute for* δ: For each number $\delta_1 > 0$ there exists a number $\delta(\delta_1) > 0$ such that $|\delta(p, q)| < \delta(\delta_1)$ implies $\delta_1(p, q) < \delta_1$.

IV_1. δ_1 is *locally of limited contraction:* For each polygon that is sufficiently small in the geometric sense the variational length is not much smaller than the variational chord. By polygon we mean a finite ordered set $P = \{p_1, p_2, \ldots, p_n\}$, by the variational length of P the number $\lambda_1(P) = \Sigma\delta_1(p_i, p_{i+1})$, by the variational chord of P the number $\mu_1(P) = \delta_1(p_1, p_n)$, by the geometric diameter $\delta(P)$ the largest of the numbers $\delta(p_i, p_j)$. Then the assumption IV_1 reads as follows: For each number $\xi_1 > 0$ there exists a $\delta(\xi_1) > 0$ such that $\delta(P) < \delta(\xi_1)$ always implies $\lambda_1(P) \geq \mu_1(P) - \xi_1|\mu_1(P)|$.

Neither δ nor δ_1 is required to be symmetric or non-negative. Some pairs of points may have positive, other pairs negative distances, as is actually the case in the applications to indefinite problems of the calculus of variations. Neither δ nor δ_1 is required to satisfy the triangular inequality or, what is equivalent, the condition "Length not smaller than chord for any polygon." For δ we merely assume the much weaker condition II, for δ_1 the condition IV_1 "Length not *much* smaller than chord for *sufficiently small* polygons." We do not require $\delta_1(p, q) \neq 0$ for distinct points.[2]

By a curve $C = p[\alpha, \beta]$ we mean the association of a point $p(\gamma)$ with each number γ of a closed interval $[\alpha, \beta]$ such that $\lim \gamma_n = \gamma$ implies $\lim \delta(p(\gamma_n), p(\gamma)) = 0$. We call $P = \{p_1, p_2, \ldots, p_n\}$ a subpolygon of C if $p_i = p(\gamma_i)$ where $\alpha = \gamma_1 < \gamma_2 < \ldots < \gamma_n = \beta$. We set $\nu_C(P) = \text{Max.}(\gamma_{i+1} - \gamma_i)$. A sequence of subpolygons $P_1, P_2, \ldots$ of C will be called distinguished if $\lim \nu_C(P_n) = 0$. For each distinguished sequence we form $\limsup \lambda_1(P_n)$ and call $\bar{\lambda}_1(C)$ or upper variational length of C the least upper bound (l.u.bd.) of these numbers. A distinguished sequence $P_1, P_2, \ldots$ for which $\lim \lambda_1(P_n) = \bar{\lambda}_1(C)$ will be called a maximal sequence of C. Besides the variational length $\lambda_1(P)$ of a polygon $P = \{p_1, p_2, \ldots, p_n\}$ we form its absolute variational length $|\lambda_1|(P) = \Sigma|\delta_1(p_i, p_{i+1})|$.

Existence and semicontinuity theorem. Under the assumptions I, II, III_1, IV_1 let C *be a curve containing a maximal sequence* $P_1, P_2, \ldots$ *for which*[3]

$$\text{l.u.bd.}\ \frac{|\lambda_1|(P_n)}{\lambda_1(P_n)} < \infty \text{ if } \bar{\lambda}_1(C) \neq 0, \qquad \text{and}$$

$$\text{l.u.bd.}\ |\lambda_1|(P_n) < \infty \text{ if } \bar{\lambda}_1(C) = 0.$$

Then there exists a finite or infinite number $\lambda_1(C)$ *such that we have lim* $\lambda_1(Q_n) = \lambda_1(C)$ *for each distinguished sequence* $Q_1, Q_2, \ldots$ *of subpolygons of* C. *The functional* $\lambda_1(C)$ *is l.s.c.*, that is to say, if $C_1, C_2, \ldots$ is a sequence of curves which geometrically converge toward C, i.e., whose Fréchet distances from C based on the geometric δ-distance converge toward 0, then lim inf $\lambda_1(C_n) \geq \lambda_1(C)$.[4] *Moreover* $\lambda_1(C)$ *is additive along each curve* C *for which*

$\lambda_1(C)$ *is finite*, i.e., if the segment C' of $C = p[\alpha, \beta]$ is the sum of two contiguous segments C_1 and C_2, say $C_1 = p[\alpha', \gamma]$, $C_2 = p]\gamma, \beta']$, $C' = p[\alpha', \beta']$ where $\alpha \leq \alpha' \leq \gamma \leq \beta' < \beta$, then $\lambda_1(C') = \lambda_1(C_1) + \lambda_1(C_2)$.

3. *The Variational Length of Curves of Bounded Length of Comparison.*—In the calculus of variations we have to consider line integrals that are 1) l.s.c. only in the class of those curves whose Euclidean lengths have a common finite bound, 2) not too small when compared with the Euclidean length of the curve along which they are taken. An analysis of the proofs shows that this length with which the line integral is compared need not be derived from the geometric distance. We may introduce a length of comparison $\lambda_2(C)$ derived from any third distance $\delta_2(p, q)$. In the following we shall call $\delta_2(p, q)$ the *distance of comparison.* $\delta_2(p, q)$ need not be $= \delta(p, q)$ nor $= \delta_1(p, q)$. If $\delta_2(p, q)$ satisfies III_2 and IV_2, i.e., if δ_2 is an upper substitute for δ and locally of limited contraction, then the existence and semicontinuity theorem holds for the length of comparison $\lambda_2(C)$. Concerning the variational distance $\delta_1(p, q)$ we now assume III_1 and (instead of IV_1)

IV_{12}. δ_1 *is locally of limited contraction for polygons of small length of comparison:* For each $\xi_1 > 0$ and $\xi_2 > 0$ there exists a $\delta(\xi_1, \xi_2) > 0$ such that $\delta(P) < \delta(\xi_1, \xi_2)$ always implies

$$\lambda_1(P) \geq \mu_1(P) - \xi_1 |\mu_1(P)| - \xi_2[|\mu_2(P)| + |\lambda_2|(P)].$$

Second semicontinuity theorem. From I, II, III_2, IV_2, III_1, IV_{12} it follows that for each number $\lambda_2 < \infty$ the functional $\lambda_1(C)$ is l.s.c. on the set of all curves for which $\lambda_2(C) \leq \lambda_2$.

A third theorem derives the conclusion of the second even if IV_{12} is weakened: We may admit *0-sets of exceptional points* such that polygons beginning with one of these points may be as much contracted as we please provided that in the neighborhood of these points all closed polygons have a non-negative variational length.

4. *The Class of Curves of Bounded Length of Comparison.*—We now make two further assumptions about δ_2, the first of which implies, in particular, that δ_2 is essentially positive, viz.:

V_2. δ_2 *separates distinct points positively*, i.e., for any two distinct points p and q the greatest lower bound of the numbers $\lambda_2(P)$ for all polygons P joining p and q is > 0.

VI_2. *The space is locally connected with regard to* λ_2, i.e., each point p can be joined with each point whose geometric distance from p is sufficiently small by a curve C for which $\lambda_2(C)$ is arbitrarily small.

From III_2–VI_2 it follows that in a compact space for each number $\lambda_2 < \infty$ the set of all curves C for which $\lambda_2(C) \leq \lambda_2$, is compact. The lower semicontinuity and additivity of $\lambda_2(C)$ which by virtue of the existence theorem follow from III_2 and IV_2, together with V_2 and VI_2, allow us to apply a theorem on functionals of curves[5] generalizing Hilbert's theorem that in a

compact space the set of all curves whose lengths have a common finite bound is compact.

5. *A Theorem of General Analysis.—Let* β *be a function defined* (though not necessarily finite) *on the limit class* L, *and* α *a function defined on a subset* L_α *of* L *containing, in particular, all elements* e *of* L *for which* $\beta(e) < \infty$. *If* α *and* β *satisfy the conditions*

(1) *For each number* $\alpha_0 < \infty$ *the function* β *is bounded above on the set of all elements* e *for which* $\alpha(e) \leq \alpha_0$,

(2) *For each* $\beta_0 < \infty$ *the set of all elements* e *for which* $\beta(e) \leq \beta_0$, *is compact,*

and if K *is a subset of* L_α *closed in the set of all* e *for which* $\beta(e) < \infty$, *then* K *contains at least one element* e_0 *that is limit of a minimizing sequence for* α *on* K, *i.e., a sequence* $e_1, e_2, \ldots$ *for which* $\alpha(e_1), \alpha(e_2), \ldots$ *converge toward the greatest lower bound of* α *on* K.

If in addition to (1) *and* (2) α *and* β *satisfy*

(3) *For each* $\beta_0 < \infty$ *the function* α *is l.s.c. on the set of all* e *for which* $\beta(e) \leq \beta_0$,

then each element e_0 *that is limit of a minimizing sequence for* α *on* K *actually minimizes* α *on* K.

This theorem[6] is implicitly contained in the work of Tonelli who applies it to the case that L is the limit class of all curves of the plane, $\alpha(C)$ a line integral along C, $\beta(C)$ the Euclidean length of C. Applying the theorem to $\alpha(C) = \lambda_1(C)$, $\beta(C) = \lambda_2(C)$ we see that the conditions (2) and (3) are satisfied. In order to guarantee (1) we assume

VII_{12}. *The variational length is not too small compared with the length of comparison,* i.e., for each number $\lambda_1 < \infty$ the function $\lambda_2(C)$ is bounded above on the set of all C for which $\lambda_1(C) \leq \lambda_1$.

From I, II, III_2–VI_2, III_1, IV_{12}, VII_{12} *it follows that in a compact space each closed class* K *of curves with finite length of comparison* λ_2(C) *contains a limit element of a minimizing sequence for the variational length* λ_1(C) *on* K, *and that each such limit element actually minimizes the variational length on* K.

6. *Applications to the Calculus of Variations.*—In the calculus of variations we deal with a geometric distance $\delta(p, q)$ satisfying the postulates of a metric space in the sense of Fréchet, and a function $\varphi(p, q)$, the integrand. As the variational distance of p and q we consider the number $\varphi(p, q) \cdot \delta(p, q)$. Since S is a metric space conditions I and II are satisfied. Condition III_1 holds whenever the integrand φ is a bounded function and, in addition to that, for many unbounded integrands. If S is a metric space in which directions are defined, and if $\varphi(p, q) = \varphi(p, q')$ whenever the directions from p to q and from p to q' are identical, then φ can be considered as a function of a point p and a direction ϑ. This is the case in the classical problems. They deal with a domain in a Euclidean space or a manifold and a function $\varphi(p, \vartheta)$ of a point and a direction. The semicontinuity and

quasiregularity conditions concerning φ formulated in the author's previous papers[7] guarantee the condition IV_1 for the variational length mentioned above, or the condition IV_{12} connecting this variational length with the length of comparison $\lambda_2(C)$ derived from the distance $\delta_2(p, q) = \delta(p, q)$, i.e., with the geometric length. In this way the results of the previous papers subsume under the theory of length developed in the present paper. Moreover we see that instead of comparing the line integral (the variational length) with the ordinary Euclidean length we may compare it with any line integral of comparison derived from a distance $\delta_2(p, q)$ that satisfies the conditions III_2–VI_2.

[1] It would be sufficient to make topological assumptions concerning the underlying limit class. See [4].

[2] Nor have we to assume for the proofs of the theorems on length that $\delta(p, q) \neq 0$ if $p \neq q$. But this assumption is necessary if we wish to guarantee that the curves of the metric theory are continuous curves in the sense of topology.

[3] I am indebted to A. N. Milgram for suggestions in connection with this condition. It is necessitated by the admission of variational distances of both signs, and corresponds to the concept of absolute convergence in the theory of series. The condition may also be formulated in the following way: If $\bar{\lambda}_1(C) < \infty$, then l.u.bd. $|\lambda_1|(P_n) < \infty$; if $\bar{\lambda}_1(C) = \infty$, then l.u.bd. $\dfrac{|\lambda_1|(P_n)}{\lambda_1(P_n)} < \infty$.

[4] Let $\delta'(p, q)$ be any normal and uniformly continuous distance for which $\delta(p, q)$ is an (upper and lower) substitute, i.e., such that $|\delta(p, q)|$ is as small as we please provided that $|\delta'(p, q)|$ is sufficiently small. Then by replacing the geometric δ-distance by the δ'-distance we do not affect the existence, the numerical value or the semicontinuity of $\lambda_1(C)$. Essentially, we deal thus with *line integrals under topological conditions*.

In order to get an upper semicontinuous functional $\lambda_1(C)$ we have to make assumptions dual to III_1 and IV_1.

[5] The theorem and the conditions V and VI are contained in the author's paper in *Ergebnisse eines mathematischen Kolloquiums 8*, Vienna (1937), p. 22. The local connectedness with regard to λ_2 should not be confounded with the *F*-local connectedness recently introduced by Morse.

[6] See the paper quoted in [5] p. 13. We shall denote by (4) the condition that for any convergent sequence of elements $e_1, e_2, \ldots$ for which $\lim \alpha(e_n) = \infty$, we have $\lim \beta(e_n) = \infty$. Then we can supplement the principle of Tonelli by the following statement: In presence of the conditions (2) and (4) the condition (1) is equivalent with the following condition: Each sequence $e_1, e_2, \ldots$ for which there exists a number β' such that $\beta(e_n) \leq \beta' < \omega$ for all n, contains a converging subsequence.

[7] See the paper quoted in [5] and *Proc. Nat. Acad. Sci.*, **23**, 246–248 (1937).

GENERAL TRIHORNOMETRY OF SECOND ORDER[1]

BY EDWARD KASNER AND JOHN DE CICCO

DEPARTMENTS OF MATHEMATICS, COLUMBIA UNIVERSITY AND BROOKLYN COLLEGE

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In this paper, which is a continuation of the paper by the authors, "Conformal Geometry of Horn Angles of Second Order," these PROCEEDINGS, **24,** 393–400 (1938), we shall give the laws connecting the *nine* conformal invariants of a trihorn of second order.[2] We shall consider only *general* trihorns (as defined below).

Instead of letting (x, y, z) denote the first three derivatives of the curvature with respect to the arc length (as was done in the preceding paper), we shall find it more convenient to define (x, y, z) by the equations

$$x = 2\gamma', \quad y = \sqrt{5}\gamma'', \quad z = 2(\gamma''' - \gamma^2\gamma'), \tag{1}$$

where $(\gamma', \gamma'', \gamma''')$ are the first three derivatives of the curvature γ with respect to the arc length of any curve C of the horn-set (γ) at the common point. A horn-set (γ) can be regarded as a three-dimensional space, called the K_3-*space*, where any point of K_3 is a curve $C(x, y, z)$ of the horn-set (γ). The group of conformal transformations of the plane induces a special affine five-parameter group G_5 between the K_3-spaces.[3]

A *general line* (*linear series*) consists of the ∞^1 points of a K_3-space which satisfy two linear equations of the form $y = px + r$, $z = qx + s$, where p, q, r, s are constants such that $q - p^2 \neq 0$. A *general plane* (*flat congruence*) is composed of the ∞^2 points which satisfy a single linear equation of the form $z = ax + by + c$, where a, b, c are constants such that $4a + b^2 \neq 0$.

Under the group G_5, two points (a horn angle of second order) of a given K_3-space possess the fundamental invariant

$$M_{12} = \frac{(x_2 - x_1)^3}{(x_2 - x_1)(z_2 - z_1) - (y_2 - y_1)^2}, \quad M_{21} = -M_{12}; \tag{2}$$

also the sign of $x_2 - x_1$ is invariant. We call M_{12} the *distance* between any two points of the K_3-space.

Two intersecting general lines of a given K_3-space possess the two unique independent quantities

$$\alpha_{12} = \frac{q_2 - p_2^2}{(p_2 - p_1)^2}, \qquad \alpha_{21} = \frac{q_1 - p_1^2}{(p_2 - p_1)^2}. \tag{3}$$

We call $\alpha_{12}(\alpha_{21})$ the *direct* (*reverse*) *dihorn angle* between the ordered pair of general lines (L_1, L_2).

A *general* triangle (trihorn of second order) consists of three points (C_1, C_2, C_3) *with* $x_1 < x_2 < x_3$ such that they determine a general plane, and the three sides $L_1 = (C_2, C_3)$, $L_2 = (C_3, C_1)$, $L_3 = (C_1, C_2)$ are general lines. A general triangle possesses nine finite non-zero conformal invariants: the three distances M_{12}, M_{23}, M_{31}, the three direct dihorn angles α_{12}, α_{23}, α_{31} and the three reverse dihorn angles α_{21}, α_{32}, α_{13}. We shall state the laws involving these quantities without giving the proofs.

THEOREM 1. *The ratio* α_{ji}/α_{ki} *is always positive. The four dihorn angles* α_{ji}, α_{ki}, α_{ij}, α_{kj} *are connected by the single equation*

$$(-1)^i \left(\frac{\alpha_{ji}}{\alpha_{ki}}\right)^{1/2} + (-1)^j \left(\frac{\alpha_{ij}}{\alpha_{kj}}\right)^{1/2} = -1. \tag{A}$$

THEOREM 2. *The four dihorn angles* α_{ij}, α_{jk}, α_{ji}, α_{kj} *satisfy the single relation*

$$(-1)^j \left(\frac{\alpha_{ij}}{\alpha_{kj}}\right)^{1/2} = -\frac{\alpha_{ij} - \alpha_{ji} - 1}{\alpha_{kj} - \alpha_{jk} - 1}. \tag{B}$$

THEOREM 3. *The four dihorn angles* α_{ij}, α_{jk}, α_{ik}, α_{ji} *are connected by the single relation*

$$1 + \alpha_{ik} = \left[1 + (-1)^k \left(\frac{\alpha_{ik}}{\alpha_{jk}}\right)^{1/2}\right]\left[1 + \alpha_{ij} + (-1)^k \alpha_{ji} \left(\frac{\alpha_{ik}}{\alpha_{jk}}\right)^{1/2}\right]. \tag{C}$$

THEOREM 4. *The four dihorn angles* α_{ij}, α_{jk}, α_{ki}, α_{ji} *satisfy the single equation*

$$\alpha_{ik} = -\left[1 + (-1)^i \left(\frac{\alpha_{ki}}{\alpha_{ji}}\right)^{1/2}\right]\left[1 + \alpha_{kj} + (-1)^i(\alpha_{kj} - \alpha_{ki})\left(\frac{\alpha_{ji}}{\alpha_{ki}}\right)^{1/2}\right]. \tag{D}$$

Theorems 1, 2, 3 and 4 are the analogues of the angular identity of a triangle in ordinary plane geometry. In a general triangle, every three of the six dihorn angles are independent, but every four are related by a single formula of the type (*A*), (*B*), (*C*) or (*D*).

THEOREM 5. *The Law of Sines. The nine parts of a general triangle are connected by the equations*

$$\frac{M_{12}\left|(\alpha_{12} - \alpha_{21})^2 + 2(\alpha_{12} + \alpha_{21}) + 1\right|^{5/2}}{\alpha_{12}\alpha_{21}} = \frac{M_{23}\left|(\alpha_{23} - \alpha_{32})^2 + 2(\alpha_{23} + \alpha_{32}) + 1\right|^{5/2}}{\alpha_{23}\alpha_{32}} = \frac{M_{13}\left|(\alpha_{31} - \alpha_{13})^2 + 2(\alpha_{31} + \alpha_{13}) + 1\right|^{5/2}}{\alpha_{31}\alpha_{13}}. \tag{E}$$

The three expressions of the form $(\alpha_{ij} - \alpha_{ji})^2 + 2(\alpha_{ij} + \alpha_{ji}) + 1$ *are finite, non-zero quantities of the same sign.*

THEOREM 6. *The Law of Cosines (Type I). The five parts* M_{ij}, M_{jk}, M_{jk}, α_{ki}, α_{ik} *satisfy the single relation*

$$M_{ki} = \frac{-(\alpha_{ik}M_{ij} + \alpha_{ki}M_{jk})^3}{\alpha^3_{ik}M^2_{ij} + \alpha^3_{ki}M^2_{jk} + \alpha_{ik}\alpha_{ki}(1 + \alpha_{ik} + \alpha_{ki})M_{ij}M_{jk}}. \quad \text{(F)}$$

THEOREM 7. *The Law of Cosines (Type II). The five parts* M_{ij}, M_{jk}, M_{ki}, α_{ij}, α_{kj} *are connected by the single relation*

$$M_{ki} = \frac{-M_{ij}M_{jk}\left[1 + (-1)^j\left(\frac{\alpha_{kj}}{\alpha_{ij}}\right)^{1/3}\right]^2\left[\alpha_{ij} - (-1)^j\left(\frac{\alpha_{ij}}{\alpha_{kj}}\right)^{1/3}\right]}{\alpha_{kj}M_{jk} + \alpha_{ij}M_{ij}}. \quad \text{(G)}$$

THEOREM 8. *The Law of Cosines (Type III). The five parts* M_{ij}, M_{jk}, M_{ki}, α_{jk}, α_{ji} *satisfy the single relation*

$$M_{ki} = \frac{-\alpha_{jk}\alpha_{ji}M_{jk}M_{ij}[(\alpha_{ji}M_{jk})^{-1/3} + (\alpha_{jk}M_{ij})^{-1/3}]^2}{(1 + \alpha_{jk})(\alpha_{ji}M_{jk})^{1/3} + (1 + \alpha_{ji})(\alpha_{jk}M_{ij})^{1/3}}. \quad \text{(H)}$$

We note that there is a last Type IV of Law of Cosines (Theorem 9). This is the formula (I) connecting the five parts M_{ij}, M_{jk}, M_{ki}, α_{ij}, α_{jk}. We do not write this as it is a very complicated expression. It is obtained as the eliminant with respect to a dihorn angle of the Laws of Cosines of Types II and III.

In conclusion, we observe that every four parts of a general triangle (of which at most three are dihorn angles) are independent, but every five parts are connected by a relation which is a consequence of the above stated laws.

[1] Presented to the American Mathematical Society, September, 1939.

[2] Kasner, "Conformal Geometry," *Proc. Fifth Internat. Cong. Math.*, **2**, 81 (1912); "The Two Conformal Invariants of Fifth Order," *Trans. Amer. Math. Soc.*, **44**, 25–31 (1938); and "Geometry of Conformal Symmetry (Schwarzian Reflection)," *Ann. Math.*, **38**, 873–879 (1938).

[3] As indicated by Comenetz, this may be generalized to a riemannian surface. (See his paper, "Conformal Geometry on a Surface," *Ann. Math.*, **39**, 863–871 (1938).) A horn-set (γ) consists of all curves on a riemannian surface Σ which pass through a common point in a common direction, and which possess the same geodesic curvature γ. In (1), let x and y represent the same quantities, but let z denote the expression $2[\gamma'' - (\gamma^2 + K)\gamma']$ where K is the gaussian curvature of Σ at the common point. Any horn-set (γ) of Σ may be regarded as a three-dimensional space K_3, where any point of K_3 is a curve $C(x, y, z)$ of the horn-set (γ). The *same* group G_5 between the K_3-spaces is induced by the group of conformal transformations between all riemannian surfaces.

GROUPS WHICH CONTAIN LESS THAN TEN PROPER SUBGROUPS

BY G. A. MILLER

DEPARTMENT OF MATHEMATICS, UNIVERSITY OF ILLINOIS

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The groups which separately contain less than eight proper subgroups were considered in a preceding article of these PROCEEDINGS, **25,** 367–371 (1939). If an abelian group contains exactly eight proper subgroups its order cannot be divisible by three distinct prime numbers because if one and only one of its factors were then divisible by a square the number of these cyclic subgroups would be ten and if none would be divisible by a square there would be only six proper subgroups. If the abelian group G contains exactly eight proper subgroups and its order is divisible by two distinct primes this order is of the form $p_1p_2^4$, where p_1 and p_2 are distinct primes, when G is cyclic, and when G is non-cyclic it is the direct product of the four group and a group of an arbitrary odd prime order. If the order of G is a power of a prime number it is of the form p^9, p being any prime number whenever it is cyclic. When it is non-cyclic it is one of the following two special groups: The non-cyclic group of order 49, the group of order 27 and of type 2,1. Hence there are exactly five abelian groups which separately contain eight proper subgroups.

If a non-abelian group G contains exactly eight proper subgroups then at least one of these subgroups is invariant under G since G cannot be a simple group. If only one of the proper subgroups of G is invariant and the other seven are conjugate under G there are two possible groups which result from the following theorem: *There is one and only one group of order* pk^α, p *being an odd prime number and* k *being a prime divisor of* $\mathrm{p}-1$, *which contains exactly* p *conjugate non-invariant subgroups whenever these subgroups are not all relatively commutative.* This theorem results directly from the fact that such a group has a $k^{\alpha-1}$, 1 isomorphism with a transitive group of degree p which has exactly p non-invariant subgroups. The order of this transitive group is therefore pk since it contains one and only one subgroup of order p. When $p = 7$ the two possible groups are of orders 14 and 21, being, respectively, dihedral and semi-metacyclic. In general this group contains $p + 2\alpha - 1$ proper subgroups.

If only one of the eight proper subgroups of G is invariant and the other seven are transformed under G according to an intransitive group its transitive constituents could not be of degrees 2, 5 since G contains only one invariant subgroup. If they are of degrees 3 and 4, respectively, G may be the tetrahedral group of degree 7. It could be no other group since it is supposed to contain one and only one invariant proper subgroup and

hence the intransitive group of degree 7 according to which its non-invariant subgroups would be transformed could not involve more than one proper invariant subgroup and the order of G evidently would exceed 6. If G would contain two and only two invariant proper subgroups its six non-invariant proper subgroups could not be transformed transitively since the number of invariant subgroups of a transitive group of degree six increased by the number of its other cyclic subgroups of this degree exceeds 2.

Suppose that the six non-invariant proper subgroups of G are transformed under G according to an intransitive substitution group in which the number of invariant subgroups increased by the number of the other cyclic subgroups of degree 6 does not exceed two. If each of the constituents would be of degree 2 the order of this intransitive group could not exceed 2 since it would have more than two invariant subgroups if its order was larger. If it were of order 2 it would be of degree 6 and hence G would again contain more than two invariant subgroups. The intransitive group of transformation could not have two transitive constituents of degree 3. As it also could not have two transitive constituents of degree 2 and 4, respectively, it results that G could not involve exactly two proper invariant subgroups.

If G contains three proper invariant subgroups and its five non-invariant subgroups are transformed transitively it may be the dicyclic group of order 20 according to the italicized theorem noted above. In this case $p = 5$ and $\alpha = 2$. This could be no other group since the five non-invariant subgroups would have to be transformed according to the dihedral group of degree 5. The five non-invariant subgroups of G could not be transformed under G according to an intransitive group since the order of this group could not be 12 because G contains only three invariant subgroups. It could not be 6 for the same reason. Hence there is one and only one group which has exactly eight proper subgroups including three invariant ones.

When G contains exactly four proper invariant subgroups its four non-invariant subgroups cannot be transformed under G according to a transitive group of degree 4. If they were thus transformed this transitive group could not be the alternating group of degree 4 since this group contains eight proper subgroups but only one of them is invariant. It could not be the octic group because this group transforms its four non-invariant subgroups intransitively. It could not be the four group because the subgroup of G corresponding to the identity of this four group would involve just four proper subgroups which would be non-invariant under G. Hence G would involve more than eight proper subgroups since its order would be at most 36. Similar remarks apply to the case when the substitution group in question would be cyclic. Hence it results that when G contains four proper invariant and four non-invariant proper subgroups it transforms these non-invariant subgroups according to an intransitive group of degree 4.

The four non-invariant subgroups of G could not be transformed under G according to the group of order 2 since the two pairs of conjugate subgroups would be relatively commutative. Hence G would involve more than eight proper subgroups. It therefore results that G transforms its four non-invariant subgroups according to the intransitive group of order 4 and contains a cyclic subgroup which involves the commutator subgroup and transforms the four non-invariant proper subgroups in pairs. That is, the octic group is the only group which has exactly eight proper subgroups of which exactly four are invariant. According to the italicized theorem noted above there is one and only one group which has eight proper subgroups, five of which are invariant, and transforms the three non-invariant and non-commutative ones transitively. If the three non-invariant subgroups are relatively commutative then G is the non-abelian group of order 27 which is conformal with the abelian group of type 2, 1. Since G could not contain exactly six proper invariant subgroups *there are exactly twelve groups which separately contain eight and only eight proper subgroups. Five of these are abelian and the other seven are non-abelian.*

At the opening of this article it was observed that if an abelian group contains exactly nine proper subgroups its order is divisible by at most two distinct prime numbers and if the order of such a group is divisible by two distinct primes it cannot be cyclic since $9 + 2$ is a prime number. If it were non-cyclic one of its Sylow subgroups would be non-cyclic. As this is impossible the order of G must be a power of a prime number when it is abelian and contains exactly nine proper subgroups. If G is non-cyclic it is of order 16 and of type 3, 1. When it is cyclic its order is p^{10}, p being an arbitrary prime number. There are therefore two and only two abelian groups which separately involve exactly nine proper subgroups. One of these is cyclic and the other is non-cyclic. We proceed to consider the non-abelian groups which separately involve exactly nine proper subgroups.

Such a group G contains at least one invariant proper subgroup since it cannot be simple. If it would contain only one invariant proper subgroup its eight non-invariant proper subgroups would be transformed under G according to a group of degree 8 which would not involve more than one cyclic subgroup generated by a substitution of degree 8 nor more than one invariant subgroup. Such a group could not be transitive since none of the fifty transitive groups of degree 8 satisfies these conditions.[1] It could not be intransitive and have four transitive constituents of degree 2 since it would involve more than one invariant subgroup if its order exceeded 2, and if its order were 2 it would contain a cyclic invariant subgroup which would transform the eight non-invariant subgroups in pairs and hence G would involve more than nine subgroups. Its transitive constituents could not be of degrees 6 and 2 because it would contain only one invariant subgroup. Its transitive constituents could not be of degree 3 and 5, respec-

tively, nor of degrees 4, 4 or of degrees 4, 2, 2. Hence it results that G would contain more than one proper invariant subgroup if it would contain exactly nine proper subgroups.

If G contained just two proper invariant subgroups its seven non-invariant subgroups could not be transformed transitively under G because the order of G could not be divisible by 7. They could also not be transformed according to an intransitive group whose transitive constituent would be of degrees 5, 2 since its order could not be divisible by 5. The degrees of the transitive constituents of such a group could not be either 3, 4 or 3, 2, 2 and hence G involves at least three invariant subgroups. If it involved exactly six non-invariant proper subgroups they could not be transformed transitively under G since G is supposed to contain only three invariant proper subgroups. They could also not be transformed according to the substitution group of order 2 and degree 6 because G would then involve more than three invariant subgroups. This would also be the case if all the transitive constitutions of this substitution group were of degree 2 and the order of the group would exceed 2.

The six non-invariant subgroups of G could not be transformed according to an intransitive group having two transitive constituents of degree 3 or one constituent of degree 4 and one of degree 2. Hence G contains at least four invariant proper subgroups and if it has exactly four such subgroups its non-invariant subgroups cannot be transformed transitively. They can also not be transformed intransitively under G because the transitive constituents would be of degree 2 and of degree 3, respectively. Hence it results that if a group contains exactly nine proper subgroups it contains at least five invariant proper subgroups. If it contains this number of invariant proper subgroups its four non-invariant subgroups cannot be transformed transitively, but they are transformed intransitively according to the intransitive group of degree 4 and order 4 when G is the dicyclic group of order 16.

The four non-invariant subgroups of G could not be transformed under G according to a substitution group of order 2 since such a group would involve an invariant cyclic group which would transform these four subgroups in pairs. If G contained six invariant subgroups and three non-invariant subgroups it could not transform these three subgroups cyclically since it contains just nine subgroups. For the same reason it could not transform the three non-invariant subgroups according to the symmetric group of degree 3. If G contains two and only two non-invariant subgroups it is known to be of order 16 and conformal to the abelian group of type 3, 1. Hence *there are four and only four groups which separately involve nine proper subgroups. Two of these are abelian and the other two are non-abelian.*

[1] G. A. Miller, *Collected Works*, **1**, 361–403 (1935).

DEVELOPMENT OF EYE-COLORS IN DROSOPHILA:[1] BACTERIAL SYNTHESIS OF v^+ HORMONE

By Edward L. Tatum

School of Biological Sciences, Stanford University

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The development of eye-color in certain insects is controlled by specific diffusible substances[2] so far found only in insects. In Drosophila two substances, v^+ hormone and cn^+ hormone, are essential for pigment formation, and are not produced by flies homozygous for the genes *v* and *cn*, respectively. It is also known[3,4] that *v bw* animals grown at low levels of nutrition become capable of producing v^+ hormone and therefore develop pigmented eyes. This so-called "starvation effect" is prevented by the addition of sugar to the diet.

Khouvine, *et al.*,[3] reported that *v bw* larvae raised on peptone-glucose media containing tryptophane developed eye pigment while those grown on tryptophane-deficient media did not. These experiments, however, were not carried out under aseptic culture conditions. Since studies of nutrition in our laboratory have shown that such media will not support growth of Drosophila larvae under aseptic conditions, it seemed probable that growth of microörganisms made Khouvine's media adequate. These microörganisms may also have been concerned in the expression of the tryptophane effect, possibly through starvation.

TABLE 1

Effect of Tryptophane and Growing *Bacillus sp.* on Eye-color of *v bw D. melanogaster*

Figures in Parentheses Give Number of Adult Flies. (1.5 Per Cent Agar Medium 10 Cc. in 35 Cc. Vials.)

Additions to Medium	Cultural Conditions	Prolongation of Larval Life in Days	Eye-color*
			2.5–3.0
	Sterile	3–5	(9)
Dry yeast, 0.5 per cent	Inoculated with *Bacillus sp.*	4	2.0–3.0 (6)
Dry yeast, 0.5 per cent and	Sterile	2–5	0.0–1.0 (27)
sucrose, 2.0 per cent	Inoculated	1–2	0.5–2.0 (17)
Dry yeast, 0.5 per cent, sucrose, 2.0 per cent and tryptophane, 10 mg. per 10 cc.	Sterile	1–6	0.0–1.0 (40)
	Inoculated	1–2	2.0–5.0 (39)

* For significance of eye-color values cf. Tatum and Beadle.[6]

Preliminary experiments designed to test the effect of tryptophane on eye-pigment development in *v bw* Drosophila under aseptic conditions, as described elsewhere,[5] gave entirely negative results. One culture, however, was accidentally contaminated with an aerobic bacillus, and a marked effect on eye-color of the flies developing in it was observed. This unidentified *Bacillus sp.* was obtained in pure culture, and the effect of its growth on the development of eye-color was then investigated more thoroughly. The results given in table 1 show that neither the bacillus nor tryptophane separately had any significant influence on pigment production. However, in the presence of both the organism and tryptophane, eye pigmentation was greatly intensified. This shows conclusively that tryptophane is able to modify eye-color only through the intermediation of microörganisms.

These experiments did not eliminate a possible action of the organisms and tryptophane through starvation of the larvae, although the sugar concentration used should inhibit the starvation effect.[5] Accordingly, another experiment was made, using a medium containing 1.5 per cent agar, 0.5 per cent yeast, 3 per cent sucrose and 10 mg. tryptophane per 10 cc. Separate cultures were inoculated with the bacilli on the first, second and third days after hatching of the larvae. Even those flies developing on the medium inoculated on the third day developed eye-pigment (34 flies, eye-color 3.5 to 5.0[6]), although calculation of the total time to eclosion indicated that the bacteria had been introduced after most of the larvae had passed the 72-hour "critical period." After this time larvae are not modified by starvation.[4] It therefore seemed probable that the observed effect on eye-color was not caused by starvation. A more nearly direct test was made by transferring 72-hour-old, sterile, fully fed *v bw* larvae[5] to a 3-day-old culture of the *Bacillus sp.* growing on dry yeast sucrose-agar with tryptophane. Larvae over 72 hours old cannot be influenced by starvation. Nevertheless, the adult flies from this experiment showed definitely modified eye-color (22 flies, eye-color 1.0 to 3.5). In another similar experiment, the *Bacillus sp.* was grown on yeast sucrose-agar with tryptophane and the culture was autoclaved after 3 days. Fully fed, 72-hour-old, sterile *v bw* larvae were then placed aseptically on this autoclaved medium. The flies eclosed in normal time (215 hours from egg-laying) and were strongly modified, 36 flies, eye-color 3.0 to 3.5. Corresponding tests showed that *su*^{2}*-v, v;bw* flies were also strongly modified; 10 ♂♂, eye-color 3.0 to 3.5; 13 ♀♀, eye-color 3.5 to 4.0. Normal eye-color values of this stock, ♂♂ = 1.0, ♀♀ = 2.0[6]. On the other hand, *cn bw* flies were not affected at all (15 flies, eye-color 0.0).

The eye-color modification observed in these experiments was definitely not associated with a starvation effect. The concentration of sucrose used in all experiments would have inhibited almost completely any effect of

starvation.[5] Moreover, pigmentation was also produced in larvae which were too old to be affected by starvation.[4]

It has been found[7] that starvation of *v bw* larvae modifies the fat body so that after transplantation it will induce pigmentation of the eyes of a normal *v bw* host. Fat bodies from *v bw* larvae cultured on autoclaved tryptophane medium on which the bacterium had been grown were transplanted into normal *v bw* larvae. The results showed that the fat bodies were not modified as they would have been by starvation (12 flies, eye-color 0.0).

The evidence indicates conclusively that the eye-color change is due to the ingestion by the larvae of a specific substance, with v^+ activity, which is produced by the *Bacillus sp.* only in the presence of tryptophane. The v^+ and cn^+ hormones are known to be effective when taken in through the digestive system.[8] Extracts have been prepared from the autoclaved bacterial culture and injected into *v bw* larvae. Although these extracts were quite toxic, 20 pupae developed far enough to show eye-color (1.0 to 2.5). Six eclosed flies showed eye-color ranging from 0.5 to 2.7. The modification of *v bw* flies following injection of extracts is the most specific and critical available test for v^+ eye-color hormone. The method of extraction from the agar medium showed that the substance produced by the bacteria was soluble in water and ethyl alcohol, but insoluble in acetone and chloroform and was heat-stable. It therefore behaved in the same way as does v^+ hormone obtained from Drosophila pupae.[6] Another characteristic of v^+ hormone is that it is transformed into cn^+ hormone by *v bw* flies. A preliminary test showed that the bacterial product, which has only v^+ hormone activity, was also changed into cn^+ hormone in this way. Fully fed, 72-hour-old *v bw* larvae were fed on the autoclaved bacterial culture. Forty-eight hours after pupation, 15 of these were boiled, crushed and fed[8] under aseptic conditions to five *cn bw* larvae. Four flies developed and showed a definite eye-color modification of 0.3.

It should be pointed out that the active bacterial product resembles v^+ hormone in yet another way. All active v^+ (and cn^+) hormone fractions as yet prepared from Drosophila pupae[6] have been decidedly yellow in color. The active preparations from the bacteria show the same clear yellow color. It seems probable that this color is associated with the hormone itself, since the bacteria grown in the absence of tryptophane do not produce the active substance, and the yellow color is not developed.

A number of experiments have been carried out to determine the optimal conditions for the production of the effective substance by the unidentified bacillus. The organisms were grown 3 days on each medium. After autoclaving and slanting the medium, 72-hour-old, fully fed *v bw* larvae were introduced. The results (table 2) show that the active substance is produced only under aerobic conditions (solid medium or aerated liquid

TABLE 2

PRODUCTION OF SUBSTANCES WITH v^+ ACTIVITY BY *Bacillus sp.* IN 3 DAYS OF GROWTH UNDER VARIOUS CONDITIONS

Medium: dry yeast, 1.0 per cent; sucrose, 3.0 per cent; agar, 1.5 per cent; and tryptophane, 10 mg.; total volume, 10 cc. in 35 cc. vials. Figures in body of table represent eye-color values, those in parentheses give number of adult flies.

COMPLETE CULTURE MEDIUM	BACTERIAL CELLS FROM CULTURE MEDIUM*	CULTURE MEDIUM WITH BACTERIAL CELLS REMOVED	LIQUID CULTURE MEDIUM,** NON-AERATED	LIQUID CULTURE MEDIUM,** AERATED
3.0–3.5	0.5	3.0–3.5	0.0	3.0–3.5
(25)	(15)	(17)	(19)	(3)

* Cells added to fresh medium, autoclaved and tested.

** 1.5 per cent agar added to medium just before autoclaving.

medium), and that it readily diffuses from the bacterial cells into the medium.

The influence of the tryptophane concentration was then investigated, using solid medium and growing the bacilli 3 days before autoclaving the

TABLE 3

INFLUENCE OF TRYPTOPHANE CONCENTRATION ON PRODUCTION OF SUBSTANCE WITH v^+ ACTIVITY BY *Bacillus sp.*

(Basic agar medium and significance of figures as in table 2)

TRYPTOPHANE CONCENTRATION IN MG. PER 10 CC.						
0	1	5	10	20	40	70
0.0	0.1–0.5	2.0–3.0	3.0–3.5	3.0–3.2	2.5–3.0	2.0–3.5
(25)	(30)	(22)	(27)	(25)	(19)	(21)

culture and then adding 72-hour-old *v bw* larvae. The results (table 3) show that the optimal concentration of tryptophane under these conditions was 10 mg. per 10 cc. of medium. Higher concentrations did not increase the intensity of eye-pigmentation. Presumably, the bacteria can produce only a certain amount of the effective substance in 3 days, even when provided with an excess supply of tryptophane.

The results of a series in which the bacteria were allowed to grow on a medium containing 10 mg. tryptophane per 10 cc. for different periods of time before autoclaving, showed that the eye-color intensity increased with the time of bacterial growth (table 4). All the flies developing on the me-

TABLE 4

INFLUENCE OF TIME OF GROWTH OF *Bacillus sp.* ON THE PRODUCTION OF SUBSTANCE WITH v^+ ACTIVITY

(Medium and significance of figures as in table 2)

TIME OF BACTERIAL GROWTH IN DAYS				
1	3	5	7	10
1.0–2.0	3.0–3.5	4.0–4.5	4.5–5.0	5.0
(15)	(25)	(22)	(16)	(23)

dium cultured for 10 days showed the maximum possible modification (eye-color 5.0).

The influence of cultures of known microörganisms on eye-color modification has also been tested (medium containing 1 per cent yeast, 3 per cent sucrose, 1.5 per cent agar and 10 mg. tryptophane per 10 cc., and 3 days' growth before autoclaving). Only the unidentified *Bacillus sp.* has proved effective. *B. subtilis*, *B. mesentericus*, *B. terminalis*, *B. megatherium*, *Ps. aeruginosa*, *E. coli* and *S. cerevisiae* were without effect under the experimental conditions used. It is possible, however, that under other conditions some of these organisms might also be effective.

Discussion.—The diffusible hormones which control insect eye-color development have been considered to be specific substances produced only by insects. The results described in this report show that under suitable conditions certain bacteria synthesize a substance which has v^+ hormone activity in Drosophila. The final proof of the identity or non-identity of this active substance with v^+ hormone will be possible only when both are isolated in the pure state. However, all the available evidence indicates that the chemical and biological properties of the two substances are identical.

[1] Work supported by funds granted by the Rockefeller Foundation.

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[4] Beadle, G. W., Tatum, E. L., and Clancy, C. W., *Biol. Bull.*, **75**, 447 (1938).

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[6] Tatum, E. L., and Beadle, G. W., *Jour. Gen. Physiol.*, **22**, 239 (1938).

[7] Beadle, G. W., Tatum, E. L., and Clancy, C. W. (in press).

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NUTRITIONAL REQUIREMENTS OF DROSOPHILA MELANOGASTER[1]

BY E. L. TATUM

SCHOOL OF BIOLOGICAL SCIENCES, STANFORD UNIVERSITY

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In spite of the rapid recent advance in knowledge of the nutritional and vitamin requirements of higher animals, plants and microörganisms, insect nutrition is in almost the same chaotic state as was vitamin research twenty years ago. The earlier work on insect nutrition was done before the complex nature of the B group of vitamins was known, and was often not carried out under aseptic conditions. The results obtained were therefore

inconclusive. Trager and Subbarow[2] have reviewed the significant literature, and a brief summary here will suffice.

Many if not all insects require for growth and development food materials supplied in nature by yeast or other microörganisms. There is no reason to suppose that insect larvae have different basic nutritional requirements. Some, such as the mosquito, may be slightly more exacting in their requirements than Drosophila and some other insects. Baumberger[3] showed that autoclaved yeast was a completely adequate food for Drosophila, but Hinman[4] and Trager[5] found that mosquito larvae need for growth an additional factor present in living yeast and in liver extracts.

There is no evidence or indication that insect larvae need any of the known vitamins other than those of the B group. Thiamin (B_1) and riboflavin (B_2) have been shown to be essential for mosquito larvae[2] and for Drosophila.[6] It has been suggested that nicotinic acid and vitamin B_6 may also be required by mosquito larvae.[2]

Several species of insects need certain fat-like substances in addition to these B vitamins. Hobson[7] reviewed the earlier work on this requirement and showed that the unsaponifiable fat fraction essential for Lucilia could be replaced by pure cholesterol. Van't Hoog[8] showed that cholesterol (replaceable by certain other sterols) was also required by Drosophila.

Even in the presence of these above-mentioned substances, Drosophila and mosquito larvae will not grow and develop unless yeast or liver fractions are added to the food. Trager and Subbarow[2] obtained two fractions from liver extract by treatment with barium hydroxide and alcohol. These were separately almost inactive for mosquito larvae but were effective when recombined. One of these liver factors could also be adsorbed on charcoal and eluted. Lafon[9] concluded that yeast contains one factor easily adsorbed and inactivated by hydrolysis, which accelerates growth of Drosophila larvae, as well as a second factor indispensable for growth, which is not adsorbed and is not inactivated by hydrolysis. Lafon was unable to elute the first factor from the adsorbent, but showed that it was readily utilized by the larvae when fed in the adsorbed state.

The investigation of the nutrient requirements of *Drosophila melanogaster* has been undertaken in this laboratory in connection with the study of the role of diet in eye-pigment development in this insect. The preliminary results indicate rather conclusively that yeast contains three distinct substances or groups of substances essential for Drosophila. The activity of these factors does not depend on their content of any of the known vitamins. The need of riboflavin by Drosophila larvae has been substantiated, and in addition, nicotinic acid has been found to be required for normal growth.

Materials and Methods.—The feeding experiments in this investigation were carried out under aseptic conditions, using vermilion brown (*v bw*)

Drosophila melanogaster. The eggs were collected over a 3 to 4 hour period, sterilized in alcohol as described elsewhere[10] and placed on the freshly autoclaved test media. All cultures were held at 25°C. and observed every 24 hours. After pupation of the larvae, sterility was checked by streaking a loopful of the medium on to yeast extract-sucrose agar slants. Any cultures which were not bacteriologically sterile were discarded.

All media contained 1.5 per cent purified agar. This and the other desired constituents were placed in 35-cc. vials. The final volume of medium in each vial was 10 cc. The vials were stoppered with cloth-covered cotton plugs, sterilized in the autoclave and finally the medium was slanted, agitating while cooling if necessary to keep any insoluble materials in suspension.

Preliminary Experiments.—The preliminary investigations of the nutrient requirements of Drosophila larvae centered around attempts to develop a synthetic or at least a semi-synthetic medium which would support growth under aseptic conditions. A basal medium was used which contained 0.15 per cent inorganic salts;* 0.5 per cent sucrose; and 2 per cent acid hydrolyzed casein supplemented with 2 mg. of tryptophane per 10 cc. The substances listed in table 1 were tested separately and together in this basal medium.

TABLE 1

SUBSTANCES TESTED IN THE BASAL MEDIUM AND FOUND TO BE INACTIVE FOR GROWTH OF DROSOPHILA LARVAE

SUBSTANCE ADDED	CONCENTRATIONS TESTED PER 10 CC.
Butter fat	1 to 50 mg.
Cholesterol	0.1 to 2 mg.
Lecithin	0.01 to 10 mg.
Thiamin (B_1)	2 to 20 γ
Riboflavin (B_2)	5 to 50 γ
Nicotinic acid	0.1 to 10 mg.
Adermin (B_6)[11]	1 to 10 γ
Yeast nucleic acid	1 to 100 mg.
Glutathione	0.1 to 20 mg.

Although this list includes all of the factors suggested in the literature as essential or concerned in larval growth of insects, the test larvae in most cases failed to grow and died in from 1 to 7 days. In the few cases in which larval growth did take place, the medium was found to be contaminated with microörganisms. In most of these experiments the animals were put on the test medium before hatching from the eggs. However, in a few instances 60-hour-old larvae grown on sterile medium containing 5 per cent dry yeast were transferred aseptically to the test media. Even then they did not continue to grow, indicating that essential factors present in yeast were not stored in the larvae in a quantity sufficient to permit continued growth on deficient media.

Centrifuged and filtered yeast autolysate[12] gave good larval growth both alone and when added to the basal medium. However, experiments with Difco yeast extract in 1–3 per cent concentrations as a source of yeast factors gave only very poor growth. It seemed probable that Difco yeast extract was deficient in only one yeast factor since it is known to be quite adequate for growth of most bacteria and as a vitamin source. Therefore, all of the previously mentioned substances were retested by adding them in various combinations to the basal medium together with 2 per cent Difco yeast extract. None of these substances could replace the deficient factor in Difco extract, nor were they even under these conditions limiting factors for the growth of Drosophila larvae.

The unsuccessful attempts to substitute chemically known constituents for yeast indicated rather conclusively that for growth larvae of Drosophila require, in addition to the vitamins and other substances tried, certain other factors supplied by yeast. One of these is presumably lacking in Difco yeast extract. Therefore, an effort was made to determine what yeast fractions and factors were necessary.

Yeast Fractions Required by Drosophila.—In order to determine the yeast requirements of Drosophila larvae, various fractions of yeast and yeast extracts were prepared. They were tested by adding them to a basal medium containing per 10 cc.: B_1, 2 γ; B_2, 5 γ; B_6, 10 γ; Nicotinic acid, 1 mg.; Cholesterol, 1 mg.; Lecithin, 1 mg.; Hydrolyzed casein, 0.2 g.; Tryptophane, 2 mg.; Sucrose, 0.05 g.; Inorganic salts, 0.015 g.; Agar, 0.15 g.

The numerous experiments will not be described in detail. The different yeast fractions gave, on testing, results which seem to be best explained by the assumption that Drosophila requires at least three distinct yeast factors or groups of factors for normal growth and development. These are not represented by the substances in the basal medium and are necessary in addition to any of these substances which may be required by Drosophila larvae.

The three factors have been designated arbitrarily I, II and III. A brief description of the occurrence, preparation and most convenient source of each is given in table 2. Factor I is easily adsorbed, and was contained relatively free of the other factors in the filtered and washed filter mass and yeast residue obtained in the preparation of Difco yeast extract.[13] This factor apparently corresponds to Lafon's adsorbed factor, and, similarly, it was available to the larvae in the adsorbed state, and was added to the test media in this form. Factors II and III are contained in Difco extract and may be separated by precipitation of factor III with barium hydroxide and ethyl alcohol. However, the filtrate containing factor II prepared in this way is rather toxic, and factor II may be obtained in better condition by extracting it from dry brewers' yeast with hot 95 per cent ethyl alcohol.

TABLE 2

SOURCES AND PROPERTIES OF THE YEAST FACTORS REQUIRED FOR GROWTH OF DROSOPHILA LARVAE

FACTOR	PRESENT IN	TREATMENT OF SOLUTION WITH $Ba(OH)_2$ + ETHYL ALCOHOL*	EXTRACTION OF DRY YEAST WITH HOT ETHYL ALCOHOL	BEST SOURCE
I	Dry brewers' yeast Yeast autolysate	Not precipitated	Not extracted	Filter mass[13] from preparation of Difco extract
II	Brewers' yeast Yeast autolysate Difco yeast extract	Not precipitated	Extracted	95% alcohol extract of dry brewer's yeast
III	Brewers' yeast Yeast autolysate Difco yeast extract	Precipitated	Not extracted	$Ba(OH)_2$ and alcohol precipitate* of Difco yeast extract

* Barium removed with sulfuric acid.

Factors I and III are not extracted by this treatment. Factor III is contained in the barium hydroxide and alcohol precipitated fraction from either Difco extract or fresh yeast autolysate. The separation of two essential factors by this treatment is in agreement with the results of Trager and Subbarow,[2] on the requirements of mosquito larvae.

The three groups of factors are quite distinct in their chemical and physical properties as is shown by their separation and also in their nutritional action. This is illustrated by the typical results given in table 3, which describes the effects of various combinations of the factors on the growth of Drosophila larvae on the vitamin-supplemented basal medium. Each fraction was added in an amount equivalent to 5 per cent dry yeast. It should be pointed out that the nutritive effect of any one of the possible combinations of these three factors was always the same, irrespective of the source and method of preparation of the different extracts used. None

TABLE 3

EFFECT OF YEAST FACTORS ON GROWTH OF DROSOPHILA LARVAE ON THE VITAMIN SUPPLEMENTED BASAL MEDIUM

FACTORS ADDED	GROWTH OF LARVAE	DAYS TO PUPARIUM FORMATION	DEVELOPMENT
I	None	...	.....
II	Very poor	...	.....
III	Slight	...	.....
I and II	Full size larvae in 6, 7 days	10, 11	Dead as early pupae
I and III	Full size larvae in 12 days	14–16	Dead as larvae or early pupae
II and III	Full size larvae in 9 days	10, 11	Normal adults
I, II and III	Full size larvae in 6 days	6, 7	Normal adults

Factor I = Washed filter mass from Difco extract.
Factor II = Alcohol extract of dry brewers' yeast.
Factor III = $Ba(OH)_2$ and alcohol precipitated fraction from Difco yeast extract.

of the individual factors was capable of supporting growth. In the presence of factor I, larval growth was possible when either II or III was added. With the combination of factors I and II, larval growth was almost normal in rate, and many of the larvae pupated normally but failed to develop further. With the combination of II and III, larval growth was quite slow, but pupation and further development proceeded in a normal fashion. This combination corresponds to Difco yeast extract. When all three factors were added, growth rate and development were almost normal. These results may best be explained as follows: Each factor is inactive individually. One factor (I) is not essential in the presence of the other two, but greatly accelerates the larval growth rate. In the presence of the first factor the two other factors (II) and (III) are interchangeable for the requirements of larval growth, but both are essential for complete metamorphosis and development to the adult form. It is possible that none of these fractions has yet been completely freed of the others, and that the relationships of more highly purified fractions may be still more clear cut.

TABLE 4

RIBOFLAVIN REQUIREMENT

Medium: Yeast Factors I, II and III (as in table 3) in basal medium without vitamin supplements. 50 eggs used for each series.

ADDITION TO MEDIUM	NUMBER OF ADULT FLIES	AVERAGE TIME FROM EGG LAYING TO EMERGENCE, DAYS
0	20	18
B_1, B_6 and nicotinic acid	15	21
B_2 (5 γ per 10 cc.)	38	13
B_1, B_2, B_6 and nicotinic acid	40	12

Riboflavin Requirement.—A complete study of the vitamin requirements of Drosophila larvae will be possible only when the three yeast fractions are obtained in a pure state. However, the combination of the three yeast fractions in the basal medium without the supplementing vitamins gave very slow growth. The addition of 5 γ of B_2 per 10 cc. completely restored the adequacy of this medium for growth of the larvae. (See table 4.) Any other essential vitamins were present in the yeast fractions used. This need for riboflavin is in accord with the findings of previous investigators.[2, 6]

Nicotinic Acid.—It was found that the alcohol-extracted dry yeast used for the preparation of factor II, when tested by itself, gave larval growth greatly inferior to that on unextracted dry yeast. Larval growth was somewhat slower than normal and larval mortality was quite high, particularly after the third day of growth. This deficiency was not due to the lowered factor II content, since vitamin supplements restored the nutritive value as completely as did the addition of the extracted fraction containing factor

II. The essential substance was found to be nicotinic acid. As is shown in table 5, the addition of 10 γ nicotinic acid per 10 cc. of medium containing 1 per cent of alcohol-extracted yeast restored growth to normal and prevented the larval mortality characteristic of this deficiency. It is possible that a slightly higher total nicotinic acid content is required, since the alcohol extraction was not exhaustive, and since some larvae completed development without added nicotinic acid. In these tests any other essential vitamins were contained in the alcohol-extracted yeast.

TABLE 5

NICOTINIC ACID REQUIREMENT

Medium: alcohol extracted dry yeast, 1.0%; agar, 1.5%; sucrose, 1.0%. Numerals in body of table indicate number of adult flies eclosed. (50 eggs used in each series.)

ADDITION TO 10 CC. MEDIUM	DAYS FROM EGG LAYING 10	11	12	TOTAL NUMBER OF ADULT FLIES
0	0	3	12	15
B_1, B_2, B_6 and nicotinic acid	19	25	0	44
Alcohol extract of dry yeast	24	17	1	42
2 γ nicotinic acid	2	8	3	13
10 γ nicotinic acid	15	29	0	44
100 γ nicotinic acid	20	22	0	42

So far no evidence as to the other vitamin requirements of Drosophila larvae is available, but it seems probable that those added in the basal medium are sufficient. Vitamins A, C and D are probably not essential since yeast, a complete food, contains little or none of these vitamins.

Summary.—1. Drosophila larvae require, for normal growth and development under sterile conditions, at least three factors or groups of factors normally supplied by yeast.

2. These factors are needed by Drosophila larvae in addition to their carbohydrate, amino acid, cholesterol and known vitamin requirements.

3. Factor I is primarily concerned in the acceleration of larval growth rate and is dispensable when the other two factors are supplied.

4. In the presence of I, factors II and III are interchangeable as regards larval growth.

5. In the absence of either II or III development is normal until pupation, but complete metamorphosis does not take place.

6. Nicotinic acid and riboflavin have been found to be vitally concerned in the growth of Drosophila larvae.

* Composition of salt mixture: K_2HPO_4, 25 g.; KH_2PO_4, 25 g.; $MgSO_4$, 10 g.; NaCl, 0.5 g.; $MnSO_4$, 0.5 g.; $FeSO_4$, 0.5 g.; and $CaCl_2$, 0.5 g.

[1] Work supported in part by funds granted by the Rockefeller Foundation.

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[12] Method of Orla Jensen, S.: Lactic acid bacteria (Copenhagen) 1919. The author wishes to thank Mr. M. Doudoroff for preparing this autolysate.
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CONTENTS

Page

BOTANY.—DEVELOPMENTAL CHANGES IN APICAL MERISTEMS . . . . . *By W. Gordon Whaley* 445

CHEMISTRY.—THE SYNTHESIS OF SULFONYL CHLORIDES BY CHLORINATION OF SULPHUR COMPOUNDS . . . . . *By Treat B. Johnson* 448

GENETICS.—HYBRIDIZATION AND TUMOR FORMATION IN MICE . *By C. C. Little* 452

GENETICS.—NUCLEASE ACTION, PROTEASE ACTION AND HISTOCHEMICAL TESTS ON SALIVARY CHROMOSOMES OF DROSOPHILA . . . . . *By Daniel Mazia and Lucena Jaeger* 456

STATISTICS.—ADJUSTED DEATH RATES . . . . . *By Carl R. Doering and Alice L. Forbes* 461

ASTRONOMY.—THE GRAVITATIONAL MOTION OF A PAIR OF RIGID BODIES . . . *By Theodore Eugene Sterne* 468

MATHEMATICS.—A THEORY OF LENGTH AND ITS APPLICATIONS TO THE CALCULUS OF VARIATIONS . . . . . *By Karl Menger* 474

MATHEMATICS.—GENERAL TRIHORNOMETRY OF SECOND ORDER. . . . . *By Edward Kasner and John De Cicco* 479

MATHEMATICS.—GROUPS WHICH CONTAIN LESS THAN TEN PROPER SUBGROUPS . *By G. A. Miller* 482

BIOCHEMISTRY.—DEVELOPMENT OF EYE-COLORS IN DROSOPHILA: BACTERIAL SYNTHESIS OF v^+ HORMONE. . . . . *By Edward L. Tatum* 486

BIOCHEMISTRY.—NUTRITIONAL REQUIREMENTS OF DROSOPHILA MELANOGASTER . *By Edward L. Tatum* 490